AD_____

Award Number: DAMD17-01-1-0781

TITLE: Mechanisms of a-Synuclein Aggregation and Toxicity

PRINCIPAL INVESTIGATOR: Benjamin Wolozin, M.D., Ph.D.

CONTRACTING ORGANIZATION: Loyola University Chicago
Maywood, Illinois 60153

REPORT DATE: September 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2003	Annual (1 Sep 02-31 Aug 03)

4. TITLE AND SUBTITLE
Mechanisms of a-Synuclein Aggregation and Toxicity

5. FUNDING NUMBERS
DAMD17-01-1-0781

6. AUTHOR(S)
Benjamin Wolozin, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Loyola University Chicago
Maywood, Illinois 60153

E-Mail: bwolozi@lumc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

α-Synuclein is a neurotoxic protein that aggregates to form pathological structures, termed Lewy bodies, in the brains of patients with Parkinson's disease. The aim of this proposal is to understand factors that stimulate or inhibit α-synuclein aggregation. In the second year of this proposal we investigated the sequence requirements for binding of iron to α-synuclein (Aim 1), the interaction of α-synuclein with different proteins and cofactors (Aim 2&3), and the mechanism of toxicity of α-synuclein (Aims 2 and 4). Our work has identified **key research outcomes**, including: 1. determining that the C-terminus of α-synuclein is crucial for metal binding, 2. identifying the proteasomal protein S6' as a protein that binds α-synuclein, 3. determining that β-synuclein can prevent proteasomal inhibition by α-synuclein, probably due to inhibiting binding of α-synuclein to S6', 4. determining that the E3 ubiquitin ligase, parkin, can prevent toxicity induced by α-synuclein in cell culture, 5. determining that α-synuclein induces phosphorylation of tau protein in vivo, and 6. comparing the vulnerability of C. Elegans over-expressing of α-synuclein to toxicity induced by metals, rotenone and paraquat, and determining that mutations in α-synuclein induce selective sensitivity to rotenone.

14. SUBJECT TERMS
Parkinson's Disease, Protein Aggregation, Iron, Mitochondria, Transfection, cDNA

15. NUMBER OF PAGES
90

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The goal of this grant proposal is to investigate the interaction of α-synuclein with metals. Our preliminary studies suggested that metals, such as iron, might promote synuclein aggregation. In this proposal, we sought to understand which metals interact with α-synuclein, how these metals might modulate α-synuclein aggregation, and how they might affect the interaction with other proteins relevant to Parkinson's disease. The work in the second year focused on examining the affects of α-synuclein on proteins related to iron metabolism, examining synuclein aggregation in neurons and monitoring the interaction of co-factors with α and β-synuclein.

Body:

Work from Aim 1 that was Accomplished

The remaining work from Aim 1 was completed. First, we constructed deletion constructs for α-synuclein. We produced 6 constructs. Each construct lacked a particular segment of α-synuclein:

Name	Amino Acids removed:	% Increase in IC50 for Iron at:	
		310 nM	380 nM
ΔExon3	9-42	0	25
ΔExon4	43-56	0	5
ΔExon5	56-101	0, but is not fully quenched by 1 mM Fe(II)	0
ΔExon6	102-129	0	0
ΔExon7	130-141	50	150
ΔCTF	125-141	75	0

These results indicate that iron interacts with the C-terminus of α-synuclein. The strong increase in the 380 nM peak observed with ΔExon7 suggests that conformational changes at the C-terminus are also critical for interactions (such as with Magnesium) that affect the 380 nM peak.

Work relevant to Aim 2:

In aim 2, we proposed to investigate how different metals might interact to affect α-synuclein aggregation in neurons, and how α-synuclein interacts with proteins associated with iron metabolism. Our first studies investigated the ability of magnesium to reverse α-synuclein aggregation in neurons (from the article 3 in the Appendix). We induced synuclein aggregation through two mechanisms, iron mediated aggregation and rotenone mediated aggregation. Rotenone is a complex I mitochondrial inhibitor. We observed that both iron and rotenone induced aggregation of α-synuclein (Fig. 1). However, while magnesium prevented aggregation of α-synuclein by iron (Fig. 2), magnesium did not prevent aggregation of α-synuclein by rotenone (Fig. 1). Magnesium did prevent the increase in ubiquitin conjugates induced by rotenone (Fig. 1).

Figure 1

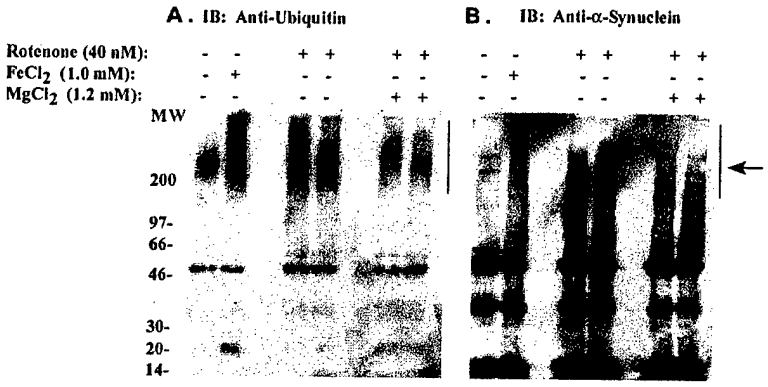


Figure 1: Induction of synuclein aggregation (right panel) and ubiquitin conjugates (left panel) by iron and rotenone. Magnesium inhibited the increase in ubiquitin conjugates but did not prevent rotenone mediated synuclein aggregation.

Figure 2

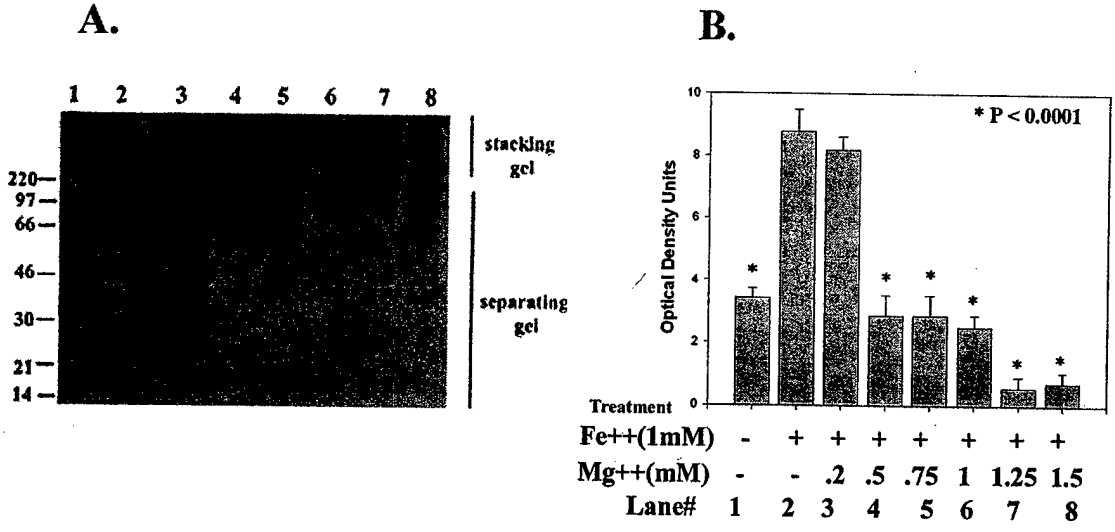


Figure 2: Inhibition of iron induced synuclein aggregation by magnesium. The concentrations of magnesium chloride corresponding to each lane in panel A are shown beneath panel B.

In the last report, I described that we are developing a nematode model of α-synuclein aggregation. We have generated C. Elegans that over-expresses α-synuclein (A53T or Wild type) driven by a neuron specific promoter. This model is coming to fruition, and we are close to submitting an article with the model. We are currently using it to screen for inhibitors of α-synuclein toxicity. Our results from this model have been quite interesting (fig. 3A & B). We have observed that α-synuclein renders the C. Elegans

differentially sensitive to rotenone toxicity (fig. 3A), but not to toxicity induced by iron, copper, manganese or cadmium (fig. 3B).

Fig. 3A

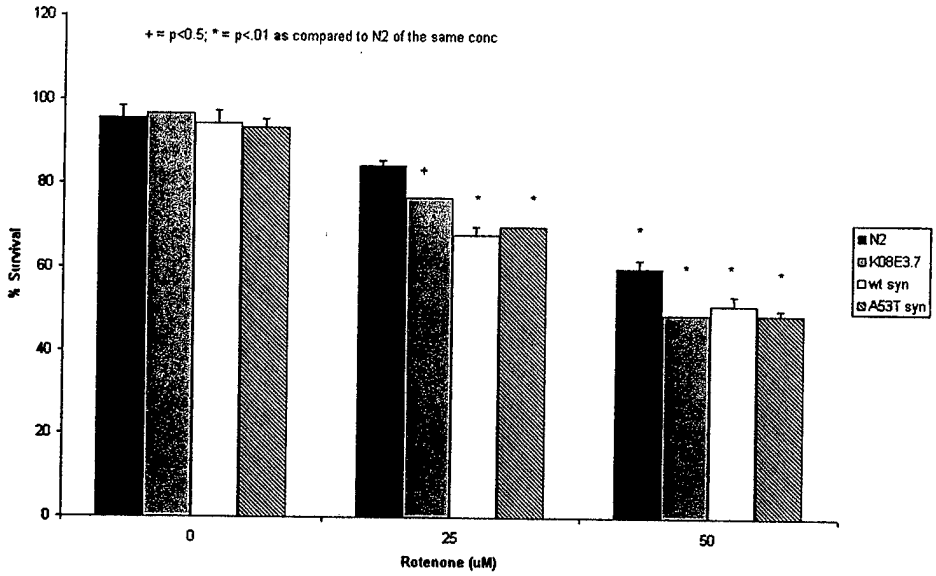


Fig. 3B

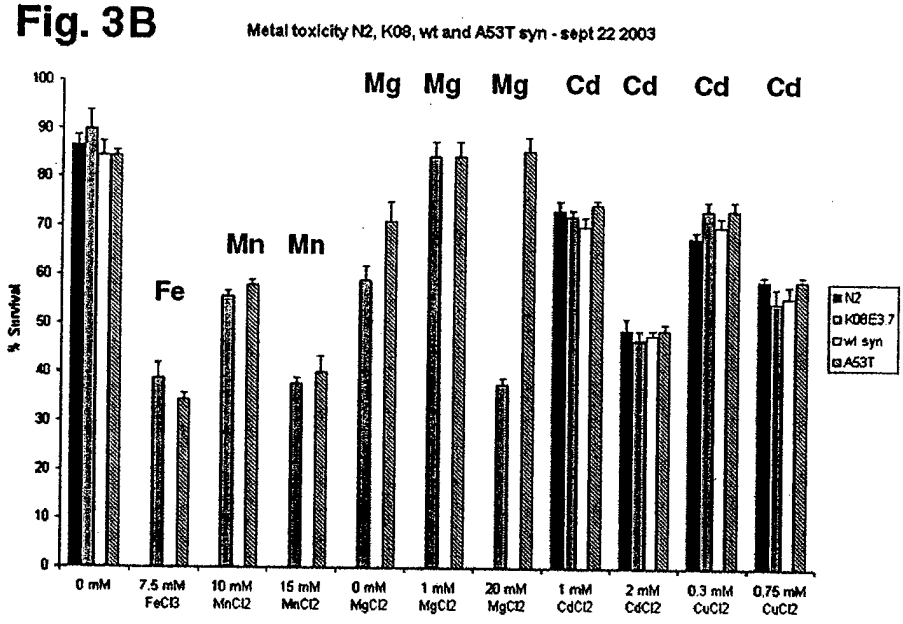


Figure 3: A. Differential rotenone toxicity of C. Elegans over-expressing α -synuclein (A53T or WT) or lacking parkin (KO8E3.7, another gene associated with Parkinson's disease) compared to non-transgenic C. Elegans (N2). B. No differential toxicity exhibited by metals in C. Elegans over-expressing α-synuclein.

We have also investigated changes in proteins related to iron metabolism associated with α-synuclein aggregation. In our initial studies, we investigated the levels of IRP1, IRP2 and ferritin, which are proteins that are directly responsive to iron levels. We sought to

determine whether α-synuclein bound these proteins or over-expressing α-synuclein changed the levels of these proteins. We found no interaction or affect of α-synuclein. Next we proceeded to examine whether α-synuclein interacted with proteins that are indirectly related to iron metabolism. Chief among these are proteins of the proteasome, because the proteasome plays a critical role in degrading proteins that have been oxidized by free radicals produced by Fe(II). We observed a number of striking findings which are described in article 1 in the appendix. We observed that α-synuclein binds the proteasomal protein S6'. Importantly, we observed that aggregated α-synuclein inhibits proteasomal function with an affinity that is 10,000 fold stronger than that of monomeric α-synuclein.

We have made a number of other important findings in work relevant to both Aims 2 and 3. It is known that α-synuclein has two homologues, β and γ-synuclein. We have observed that γ-synuclein also inhibits proteasomal function with an IC50 of approximately 100 nM. β-Synuclein does not inhibit proteasomal function, but we have made the striking observation that β-synuclein prevents the proteasomal inhibition induced by aggregated (or monomeric) α-synuclein (fig. 4). We are examining the efficacy of β-synuclein at inhibiting α-synuclein aggregation in other assays, and this could lead to an important therapeutic approach.

Fig. 4

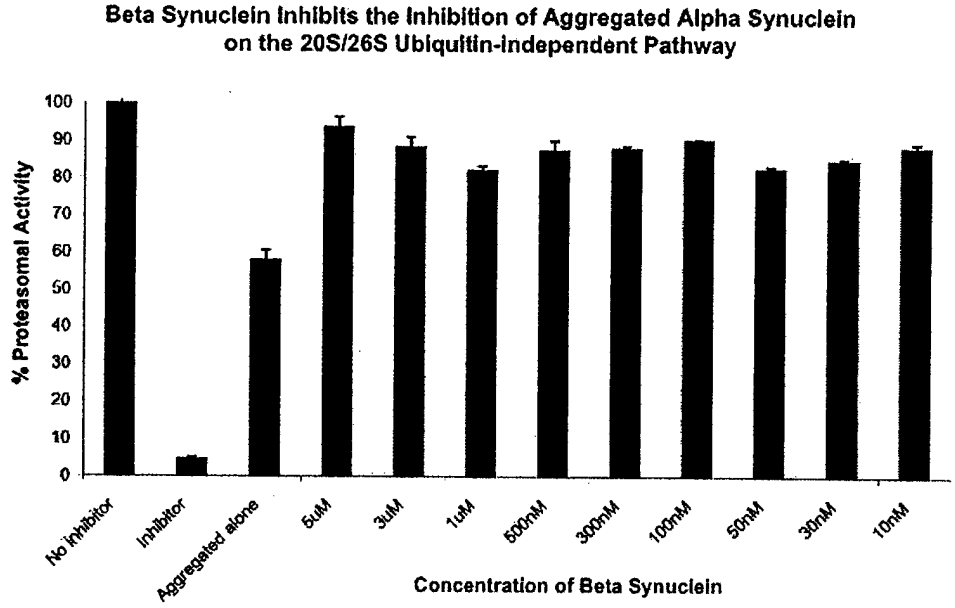


Figure 4: Aggregated α-synuclein (100 nM shown) inhibits proteasomal function, and β-synuclein is a potent antagonist of α-synuclein.

We have also made progress on other research relevant to years 3 and 4. As mentioned in the last progress report, we have established a colony of A30P α-synuclein mice. We

have now characterized the synuclein aggregation in these mice, and made the discovery that these mice develop additional pathology in the form of abnormal phosphorylation of tau protein and astrocytosis, detectable with antibodies to glial fibrilliary acidic protein. This work has been recently submitted and is contained in Appendix 6. We are currently extending this work to examine markers related to iron metabolism (Aim/Year 3) and mitochondrial metabolism (Aim/Year 4).

Key Research Accomplishments:

- Determined sequence specificity for binding of iron to α-synuclein
- Compared the efficacy of iron and rotenone in inducing α-synuclein aggregation in neurons, and demonstrated that magnesium inhibits iron induced α-synuclein aggregation but not rotenone induced α-synuclein aggregation.
- Compared the efficacy of iron and rotenone in inducing α-synuclein aggregation in neurons in C. Elegans, and demonstrated that α-synuclein shows selective toxicity to rotenone aggregation in this system.
- Demonstrated that α-synuclein binds the proteasomal protein S6'.
- Demonstrated that aggregated α-synuclein inhibits S6'/proteasomal function with a 10,000 fold higher affinity than monomeric α-synuclein.
- Examined the effects of β and γ-synuclein, and demonstrated that γ-synuclein also inhibits proteasomal function, and that β-synuclein can inhibit the actions of α-synuclein (both monomeric and aggregated).
- Analyzed α-synuclein aggregation and pathology in neurons in vivo, and demonstrated that there is an associated induction of abnormal tau pathology and gliosis.

Reportable Outcomes:

The research performed this year has resulted in publication of 5 articles relevant to α-synuclein. These articles are listed below, and are provided in the appendices.

Articles relevant to α-synuclein and the DAMD grant award that were published in last year:

1. Snyder, H., Mensah, K., Theisler, C., Lee, J. M., Matouschek, A. and **Wolozin, B.**, Aggregated and Monomeric α-Synuclein bind to the S6' Proteasomal Protein and Inhibit Proteasomal Function. J. Biol. Chem. 278:11753-9 2003.

2. Choi P., Petrucelli L., Chong M., Snyder, H. , Zhang Y., Lim K. , Chung K. , Kehoe K. , L. D'Adamio, Lee J.M., Cochran E., Bowser R., Dawson T., **Wolozin, B.**, Parkin Binds Cell Division Control-Related Protein 2 binds Parkin and Inhibits Ubiquitination. Mol. Brain Res. (in press).

3. **Wolozin, B.**, Frasier, M., Snyder, H., Choi, P. and Golts, N. Mechanisms of degeneration in Parkinson's disease. Ann NY Acad Sci. (in press).

4. Petrucelli, L., O'Farrell, C., Kehoe, K., Vink, L., Lockhart, P.J., Baptista, M., **Wolozin, B.** Choi, P., Farrer, M., Hardy, J., Cookson, M.R., Parkin protects against the toxicity associated with over-expression of synuclein: Proteasome dysfunction selectively affects dopaminergic neurons. Neuron 36:1007-19 (2002).

5. Perry G, Castellani RJ, Smith MA, Harris PL, Kubat Z, Ghanbari K, Jones PK, Cordone G, Tabaton M, **Wolozin B**, Ghanbari H., Oxidative damage in the olfactory system in Alzheimer's disease. Acta Neuropathol (Berl). (Epub ahead of print) 2003.

Conclusions:

Our results clearly demonstrate that redox active metals can induce α-synuclein aggregation both in vitro and in cell culture. However, we have now observed a significant difference between the effects of redox active metals and mitochondrial inhibitors on the aggregation of α-synuclein. This is important because it suggests that it is critical to understand the different types of pathology induced by each stressor in order to understand which type of pathology is the most relevant to PD. We have also discovered a novel mechanism of toxicity of α-synuclein, which is proteasomal inhibition, and we have discovered that β-synuclein is a potent inhibitor of proteasomal inhibition mediated by α-synuclein. We intend to examine this using other assays of α-synuclein function. This could lead the way to novel therapeutic approaches to PD.

Our results also show that magnesium inhibits α-synuclein aggregation, which suggests that magnesium might be useful in treating PD. Magnesium therapy poses a challenge for clinical application because it is unclear how much reaches the brain, and it can cause gastrointestinal distress. However, magnesium therapy is already used clinically to treat patients with preeclampsia during pregnancy.

Recommended changes: 1.) I would like permission to continue to investigate the antagonistic actions of β-synuclein towards α-synuclein. I believe that the discovery that β-synuclein antagonizes the actions of α-synuclein will provide important insights into the mechanism of toxicity of α-synuclein and could lead to important new therapeutic strategies. 2.) I would also like to continue to investigate α-synuclein aggregation in C. Elegans, as a simple *in vivo* model. We are now using this to screen other toxins relevant to PD and to screen for potential neuroproteictive agents. In addition, in Aim 4, studies in C. Elegans offer elegant methods for using genetics to determine the role of mitochondrial dysfunction in α-synuclein toxicity.

References: None

Appendices:

Articles relevant to α-synuclein and the DAMD grant award that were published in last year:

1. Snyder, H., Mensah, K., Theisler, C., Lee, J. M., Matouschek, A. and **Wolozin, B.**, Aggregated and Monomeric α-Synuclein bind to the S6' Proteasomal Protein and Inhibit Proteasomal Function. J. Biol. Chem. 278:11753-9 2003.

2. Choi P., Petrucelli L., Chong M., Snyder, H. , Zhang Y., Lim K. , Chung K. , Kehoe K. , L. D'Adamio, Lee J.M., Cochran E., Bowser R., Dawson T., **Wolozin, B.**, Parkin Binds Cell Division Control-Related Protein 2 binds Parkin and Inhibits Ubiquitination. Mol. Brain Res. (in press).

3. Wolozin, B., Frasier, M., Snyder, H., Choi, P. and Golts, N. Mechanisms of degeneration in Parkinson's disease. Ann. NY Acad. Sci. (in press)

4. Petrucelli, L., O'Farrell, C., Kehoe, K., Vink, L., Lockhart, P.J., Baptista, M., **Wolozin, B.** Choi, P., Farrer, M., Hardy, J., Cookson, M.R., Parkin protects against the toxicity associated with over-expression of synuclein: Proteasome dysfunction selectively affects dopaminergic neurons. Neuron 36:1007-19 (2002).

Articles in Review:

5. Frasier, M., Walzer, M., McCarthy, L., Magnuson, D., Lee, J.M., Haas, C., Kahle, P. and Wolozin, B., Tau phosphorylation increases in symptomatic mice over-expressing A30P α-synuclein.

Appendix 1

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 14, Issue of April 4, pp. 11753–11759, 2003
Printed in U.S.A.

Aggregated and Monomeric α-Synuclein Bind to the S6′ Proteasomal Protein and Inhibit Proteasomal Function*

Received for publication, August 22, 2002, and in revised form, January 13, 2003
Published, JBC Papers in Press, January 24, 2003, DOI 10.1074/jbc.M208641200

Heather Snyder‡, Kwame Mensah§, Catherine Theisler‡, Jack Lee‡¶, Andreas Matouschek§, and Benjamin Wolozin‡‖

From the ‡Department of Pharmacology and ¶Pathology, Loyola University Medical Center, Maywood, Illinois 60153 and the §Department of Biochemistry and Molecular Biology, Northwestern University, Evanston, Illinois 60208

The accumulation of aggregated α-synuclein is thought to contribute to the pathophysiology of Parkinson's disease, but the mechanism of toxicity is poorly understood. Recent studies suggest that aggregated proteins cause toxicity by inhibiting the ubiquitin-dependent proteasomal system. In the present study, we explore how α-synuclein interacts with the proteasome. The proteasome exists as a 26 S and a 20 S species. The 26 S proteasome is composed of the 19 S cap and the 20 S core. Aggregated α-synuclein strongly inhibited the function of the 26 S proteasome. The IC_{50} of aggregated α-synuclein for ubiquitin-independent 26 S proteasomal activity was 1 nM. Aggregated α-synuclein also inhibited 26 S ubiquitin-dependent proteasomal activity at a dose of 500 nM. In contrast, the IC_{50} of aggregated α-synuclein for 20 S proteasomal activity was > 1 μM. This suggests that aggregated α-synuclein selectively interacts with the 19 S cap. Monomeric α-synuclein also inhibited proteasomal activity but with lower affinity and less potency. Recombinant monomeric α-synuclein inhibited the activity of the 20 S proteasomal core with an IC_{50} > 10 μM, exhibited no inhibition of 26 S ubiquitin-dependent proteasomal activity at doses up to 5 μM, and exhibited only partial inhibition (50%) of the 26 S ubiquitin-independent proteasomal activity at doses up to 10 mM. Binding studies demonstrate that both aggregated and monomeric α-synuclein selectively bind to the proteasomal protein S6′, a subunit of the 19 S cap. These studies suggest that proteasomal inhibition by aggregated α-synuclein could be mediated by interaction with S6′.

A multisubunit complex, termed the proteasome, manages protein turnover in the body. Proteins can be either degraded directly by the proteasome, or they can be tagged with an 8-K_D protein, termed ubiquitin. Three different forms of the proteasome exist in a cell: the 20 S ubiquitin-independent proteasome, the 26 S ubiquitin-independent proteasome, and the 26 S ubiquitin-dependent proteasome. The 20 S particle forms the core of each form of proteasome (1, 2). Both the 26 S ubiquitin-dependent and -independent proteasomes contain the 20 S particle plus an additional smaller cap, which has a sedimentation coefficient of 19 S. Although smaller than the 20 S particle, the 19 S particle is also a multisubunit structure. The protein subunits that compose the 19 and 20 S particles are all known. The 19 S cap contains at least 18 different subunits, whereas the 20 S particle contains 28 subunits (1, 2). The protein S6′ (also known as tat binding protein 1 and Rpt5) is in the 19 S cap and is of particular interest because it was recently shown to directly bind ubiquitinated proteins, which suggests that it is required for ubiquitin-dependent proteasomal function (3).

Although the 26 S proteasome is responsible in both ubiquitin-dependent and -independent protein degradation (4, 5), the 20 S proteasome functions only in ubiquitin-independent protein degradation and is involved in 70–80% of the selective recognition and degradation of mildly oxidized proteins in the cytosol (4, 5). The 26 S proteasomal ubiquitin-dependent pathway degrades all ubiquitinated proteins within the cell and is the primary degradation pathway of the cell. The E1 ubiquitin-activating enzyme forms a thiodiester bond with mono-ubiquitin. The E2 ubiquitin-conjugating enzyme displaces the E1 enzyme and allows for conjugation of multiple ubiquitin moieties with one another. The E3 ubiquitin ligase enzyme binds both to the substrate targeted for degradation and to the E2 enzyme. The E2 and E3 enzymes are displaced, leaving a multichained ubiquitinated substrate protein that is targeted to the 26 S proteasome. This is both an ATP- and ubiquitin-dependent pathway (6–13).

Recent studies suggest that protein aggregates cause toxicity by inhibiting proteasomal function. Extended polyglutamine repeats, such as occur in mutant forms of huntingtin associated with Huntington's disease, aggregate readily (14–16). Polyglutamine aggregates inhibit ubiquitin-dependent proteasomal function (17). Aggregates of other proteins, such as the cystic fibrosis transmembrane receptor, also inhibit ubiquitin-dependent proteasomal function in cell culture (17). Many other proteins with hydrophobic domains also aggregate, and overexpressing the aggregation-prone domains of these proteins is toxic (18). The mechanism of toxicity for most aggregates is unknown.

Blockade of proteasome activity is toxic to many cell types and appears to be potentially important to many neurodegenerative diseases. Proteasomal inhibition causes apoptosis in many cell lines and is being tested as a potential chemotherapy (19). Although proteasomal inhibition causes rapid toxicity in cell culture, the slow accumulation of protein aggregates in neurodegenerative diseases might produce a correspondingly slow inhibition of the proteasome.

α-Synuclein is the major component of Lewy bodies, which are intracellular inclusions that form in Parkinson's disease

* This work was supported by National Institutes of Health Grant NS41786, United States Army Medical Research Command Grant DAMD17–01-1–0781, and the Retirement Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of, Pharmacology, Loyola University Medical Center, Bldg. 102, Rm. 3634, 2160 S. First Ave., Maywood, IL 60153. Tel.: 708-216-6195; Fax: 708-216-6596; E-mail: bwolozi@lumc.edu.

(PD)[1] (20, 21). The association of α-synuclein with Lewy bodies suggests that protein aggregation represents an important aspect of the pathophysiology of α-synuclein and of PD. The link between α-synuclein and protein aggregation has been strengthened by the discovery of mutant forms of α-synuclein, A53T and A30P, that are associated with rare cases of familial PD (22, 23). Both mutations accelerate aggregation of α-synuclein (24–27). The link between α-synuclein and aggregation suggests that understanding the mechanism of toxicity induced by protein aggregates could provide important insights into the mechanism of cell death in PD.

Native α-synuclein has been shown to bind both fatty acids and many different proteins, including phospholipase D, G proteins, synphilin-1, protein kinase C, 14–3-3 protein, parkin, and the dopamine transporter (28–34). In addition, rat α-synuclein has been shown to bind to rat S6′ (35). Of these proteins, only synphilin-1 and parkin have been identified in Lewy bodies (33, 36, 37). Perhaps because of the pleiotropic binding properties of native α-synuclein, overexpressing it in cells produces multiple cellular effects. α-Synuclein inhibits protein kinase C activity, phospholipase D activity, and the activity of the dopamine transporter, and α-synuclein has chaperone activity (28, 29, 34, 38). Overexpressing α-synuclein also inhibits proteasomal function (39). The link between α-synuclein and the proteasome is intriguing but is not directly related to the pathophysiology of Parkinson's disease, because overexpressed α-synuclein retains a native structure until the cell is subjected to a stress, such as incubation with rotenone or ferrous chloride (40–44). Thus, whether aggregated α-synuclein inhibits proteasomal function is unknown, and the mechanism by which it might inhibit the proteasome is also unknown.

In this study we examine the interaction of α-synuclein with the three different types of proteasome and demonstrate that aggregated α-synuclein binds to S6′ and inhibits ubiquitin-dependent proteasomal function.

MATERIALS AND METHODS

Cell Lines, Transfections, Chemicals, and Antibodies—The human cell line HEK 293 and the human neuroblastoma cell line BE-M17 were grown in OPTIMEM (Cell Grow) plus 10% fetal bovine serum supplemented with 200 μg/ml G418 (Sigma), as needed. G418 was used for selection. Transfections utilized FuGENE at a 3:1 ratio to DNA, 4 μg per 10-cm dish. Recombinant α-synuclein was generated using wild-type α-synuclein inserted into a ProEX-His_6 plasmid (Invitrogen) as described previously (41). Antibodies used include monoclonal anti-α-synuclein (1:1000 IB, Transduction Labs); polyclonal anti-S6′ (1:1000, Affiniti); monoclonal anti-S6′ (1:1000, Affiniti); polyclonal anti-PA700 (1:1000, Affiniti); monoclonal anti-10b (1:1000, Affiniti); polyclonal anti-α subunit 20 S (1:1000, Affiniti); and polyclonal anti-α-synuclein (against amino acids 116–131, 1:1000).

Pull-down Assay—Brain samples were precleared with nickel-agarose for one hour at 4 °C to eliminate proteins that directly bind to nickel-agarose (Invitrogen). These samples were incubated overnight with 5 μg of recombinant α-synuclein (His-tagged), either aggregated or monomeric. Samples were incubated with nickel-agarose for one hour to allow binding of the His-tagged α-synuclein (monomeric or aggregated), and then they were centrifuged at 1000 rpm for 1 min. Samples were washed three times with immunoprecipitation buffer (50 mM Tris-HCl, 10 mM EGTA, 100 mM NaCl, 0.5% Triton-X, 1 mM dithiothreitol, 1 mM protease inhibitor mixture (Sigma), pH 7.4) and run on 8–16% SDS gradient polyacrylamide gels (BioWhitaker).

Immunoprecipitations—Protein concentration was determined using BCA protein assay (Pierce), and 500 μg of each sample was used per immunoprecipitation in immunoprecipitation buffer (50 mM Tris-HCl, 10 mM EGTA, 100 mM NaCl, 0.5% Triton-X, 1 mM dithiothreitol, 1 mM proteasome inhibitor mixture (Sigma), pH7.4). Samples were precleared using protein G-Sepharose beads (Seize X, Pierce) for 1 h at 4 °C and incubated with antibody overnight at 4 °C while rocking. Samples were washed three times with immunoprecipitation buffer, resuspended in 2× dithiothreitol protein loading buffer, boiled for 5 min at 90 °C, and run on 8–16% SDS gradient polyacrylamide gels (BioWhitaker).

Aggregation of α-Synuclein—Recombinant α-synuclein incubated for 2 months at 37 °C in phosphate-buffered saline while shaking at 800 rpm; aggregation was confirmed by performing immunoblot analysis.

Immunoblot Analysis—Transfers to polyvinylidene difluoride (Bio-Rad) were done overnight at 4 °C at 0.1 A/gel in transfer buffer. The immunoblot was blocked in 0.2% I-block (Tropix) in Tris-buffered saline with 0.1% Tween 20 for one hour at room temperature while shaking. We then incubated blots overnight at 4 °C in primary antibody at appropriate concentration in 5% bovine serum albumin in Tris-buffered saline/0.1% Triton X-100. Blots were washed three times, 10 min each, and incubated three hours in secondary antibody (1:5000, Jackson Laboratories) in I-block at room temperature. Blots were washed three times and developed using a chemiluminescent reaction (PerkinElmer Life Sciences).

Sucrose Gradients—10–30% linear sucrose gradients were prepared using Hoefer SG 15 gradient maker (Amersham Biosciences) following the manufacturer's recommendations. Approximately 10 mg of monomeric or aggregated α-synuclein, as determined by BCA protein assay (Pierce), was added to the top of the gradient; they were centrifuged using a SW41 rotor for 16 ½ hours at 40,000 rpm at 20 °C. After centrifugation, 0.5-ml fractions were collected; 20 μl of each fraction was run on an 8–16% gradient gel, and immunoblotted.

In Vitro 20 S Ubiquitin-independent Chymotryptic Proteasomal Activity Assay—We incubated aggregated or monomeric α-synuclein at various concentrations with purified 20 S proteasome (human erythrocytes, BioMol) for 30 min and then added a fluorogenic substrate (Suc-LLVY-AMC, BioMol). Ten minutes later, the samples were analyzed with a GeminiXS SpectraMax fluorescent spectrophotometer (Amersham Biosciences) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

In Vitro 20/26 S Ubiquitin-independent Chymotryptic Proteasomal Activity Assay—Aggregated or monomeric α-synuclein at various concentrations was incubated with 250 μg of HEK 293 cell lysates, as determined by BCA protein assay (Pierce) in assay buffer (10 mM Tris-HCl, pH 7.8, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 5 mM ATP) for 30 or 60 min at 37 °C while shaking at 800 rpm. We then added a fluorogenic substrate (Suc-LLVY-AMC, BioMol) and incubated samples an additional 30 min at 37 °C while shaking at 800 rpm. Solutions were analyzed using an excitation wavelength of 360 nm and an emission wavelength of 460 nm with the GeminiXS SpectraMax spectrophotometer (Amersham Biosciences).

In Vitro 26 S Ubiquitin-dependent Proteasomal Activity Assay—Substrates were generated with an *in vitro* transcription and translation of substrate proteins using a T7 promoter in *Escherichia coli* lysate (Promega), supplemented with [^{35}S]methionine, and then partially purified by high-speed centrifugation and ammonium sulfate precipitation as described (45). The protease substrate for ClP assays was derived from barnase, which is a ribonuclease from *Bacillus amyloliquefaciens*; the protease substrate for proteasomal assays was derived from *E. coli* dihydrofolate reductase (DHFR) (45, 46). A ubiquitin moiety was added to the N terminus of the substrate proteins via a 4-amino acid linker from the *E. coli* lac repressor (45). Substrate proteins were constructed in pGEM-3Zf (+) vectors (Promega) and were verified by sequencing. The reaction was resuspended in 40 μl of buffer (25% (v/v) glycerol, 25 mM $MgCl_2$, 0.25 mM Tris/HCl, pH7.4) to which 5 μl of the *in vitro* reaction containing the radiolabeled ubiquitinated substrate protein was added with 35 μl of rabbit reticulocyte lysate (Green Hectares, containing 1 mM dithiothreitol) that is ATP-depleted as described (45). We incubated the reactions with and without monomeric or aggregated α-synuclein. Concentration of α-synuclein was determined by BCA protein assay (Pierce). We incubated at 37 °C for 7 min to allow initial cleavage of substrate proteins. Ubiquitination and degradation was initiated by the addition of ATP and an ATP-regenerating system (0.5 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, final concentrations). Reactions were incubated at 37 °C. At designated time points (15, 30, 45, 60, 90, 120, 150, and 180 min), small aliquots were removed and transferred to ice-cold 5% trichloroacetic acid. The trichloroacetic acid-insoluble fractions were analyzed by 10% SDS-PAGE and quantified by electronic autoradiography.

Statistics—All statistics were performed using a multifactorial analysis of variance analysis using the Statview statistical package.

[1] The abbreviations used are: PD, Parkinson's disease; DHFR, dihydrofolate reductase; DHFR-U, dihidrofolate reductase with a degradation tag; UPS, ubiquitin proteasomal system.

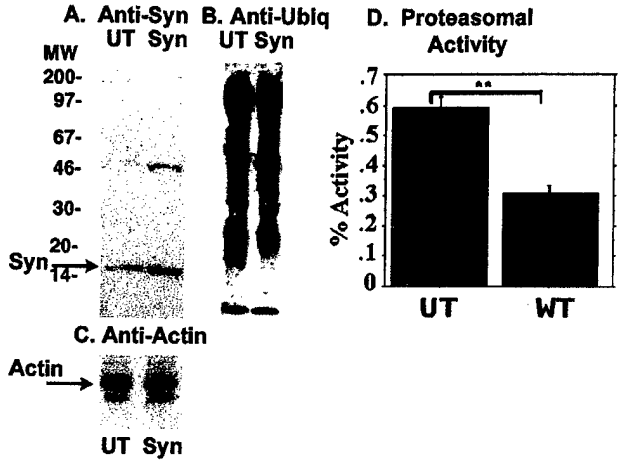


FIG. 1. **Effects of α-synuclein overexpression on the proteasomal system.** *A* and *B*, BE-M17 neuroblastoma cells were transfected with vector or wild-type α-synuclein and immunoblotted with antibodies to α-synuclein (*A*), ubiquitin (*B*), and actin (*C*). No differences in levels of ubiquitin-conjugated proteins were observed among cells transfected with vector or wild-type α-synuclein. This is a representative immunoblot from experiments that have been repeated at least three times. *D*, activity of the ubiquitin-independent proteasomal system in cell lines expressing wild-type α-synuclein compared with untransfected cells (**, $p < 0.0005$). These data represent the combined data from five experiments each containing 5 data points for each sample.

RESULTS

Overexpressing α-Synuclein Inhibits Proteasomal Degradation—To begin analyzing how α-synuclein might interact with the proteasome, wild-type α-synuclein was stably expressed in human neuroblastoma BE-M17 cells by transient transfection, and ubiquitin-dependent and -independent proteasomal activity was quantified. Because α-synuclein does not form aggregates spontaneously under these conditions, this experiment addresses whether increased concentration of cellular α-synuclein inhibits proteasomal activity. Immunoblotting of the cellular lysates demonstrated a significant increase in the α-synuclein levels in the transfected cells (Fig. 1*A*). Next, we investigated whether overexpressing α-synuclein affects the steady state levels of ubiquitin-conjugated proteins, which provides a measure of the ubiquitin-dependent proteasomal system. The BE-M17 cells expressing vector or wild-type α-synuclein were immunoblotted with anti-ubiquitin antibody. The amount of ubiquitin-conjugated proteins did not differ among the groups of transfected cells (Fig. 1, *B* and *C*).

We also investigated how overexpressing α-synuclein affects ubiquitin-independent proteasomal degradation. Previous studies report that cell lines overexpressing α-synuclein exhibit lower ubiquitin-independent proteasomal activity (39). To investigate ubiquitin-independent proteasomal activity, we measured hydrolysis rates of fluorogenic peptide analogues in cells transiently or stably overexpressing α-synuclein (Fig. 1*D*). No difference in activity was observed in cells transiently transfected with α-synuclein (data not shown). However, cell lines stably expressing wild-type α-synuclein showed an approximately 50% reduction in ubiquitin-independent proteasomal degradation, depending on the transgene (39) (Fig. 1*D*). These data suggest that α-synuclein does affect ubiquitin-independent proteasomal function.

α-Synuclein Inhibits the 20 S Proteasome—An increasing number of studies suggest that the state of α-synuclein aggregation plays a key role in the pathophysiology of PD. To better understand how α-synuclein affects the proteasome, we generated recombinant monomeric α-synuclein and aggregated α-synuclein. The aggregated α-synuclein was generated by aging recombinant α-synuclein at 37 °C for 2 months. Aggregation of α-synuclein was verified by immunoblot analysis (Fig. 2*A*). The aggregated protein ran as a smear with an average molecular weight of ~160,000 (Fig. 2*A*). The immunoblot of the aged, aggregated α-synuclein also exhibited some reactivity at 16,000. This could reflect either that the sample had some non-aggregated, monomeric α-synuclein remaining or that some of the α-synuclein could be dissociated from the aggregate by SDS. To examine this question, we fractionated monomeric or aged α-synuclein (37 °C for 2 months) by centrifugation in a sucrose gradient and immunoblotted each of the 25 fractions with anti-synuclein antibody. The initial sample used before the fractionation is shown in the first lane (Fig. 2*B*, labeled *In*). Monomeric α-synuclein had a low density and was most abundant in fractions 1–6 (Fig. 2*B*, *top*). In contrast, the aggregated α-synuclein sample showed nothing in the early fractions (corresponding to a low density) and migrated exclusively in the last fraction, suggesting a high density (Fig. 2*B*, *fraction 25*, *bottom*). Immunoblots of fraction 25 for the aggregated sample showed a small amount of SDS-sensitive α-synuclein that migrated as a monomer following exposure to SDS during the immunoblotting. This SDS-dissociable α-synuclein was particularly evident following longer exposures (Fig. 2*B*, *fraction 25B*, *bottom*). This suggests that aggregated α-synuclein contains SDS-sensitive and SDS-resistant aggregated protein.

Next, we examined the activity of purified 20 S proteasome particles in the presence of varying amounts of monomeric or aggregated α-synuclein using synthetic fluorescent peptides to monitor proteasomal activity. Increasing doses of monomeric α-synuclein progressively inhibited proteasomal activity (Fig. 3*A*). The IC_{50} for inhibition of the proteasome by monomeric α-synuclein was ~16 μM, assuming α-synuclein could achieve complete inhibition. Aggregated α-synuclein also inhibited the 20 S ubiquitin-independent proteasomal activity, exhibiting a maximal inhibition similar to that of monomeric α-synuclein (Fig. 3*B*).

To determine whether the inhibition was at the level of the proteasome or due to binding of the peptide substrate, we examined whether varying the level of substrate affected the α-synuclein-dependent proteasomal inhibition. Inhibition of the 20 S proteasome by monomeric α-synuclein increased with increasing substrate concentration (Fig. 3*C*). Increased proteasomal inhibition by α-synuclein might occur because larger effects are possible at higher rates of substrate degradation. These data indicate that the proteasomal inhibition that was observed did not result from substrate binding and substrate sequestration by α-synuclein. Thus, α-synuclein appears to inhibit the proteasomal activity via an interaction with the proteasome, rather than by binding substrate peptide.

Aggregated α-Synuclein Inhibits the 26 S Proteasome—Next, we examined the effects of monomeric and aggregated α-synuclein on a mixture of the 20 and 26 S proteasomes in HEK 293 cell lysates. Monomeric α-synuclein inhibited the 20 S/26 S proteasome mixture only partially, which could reflect greater inhibition of the 20 S proteasome complex and less inhibition of the 26 S proteasome complex (Fig. 4*B*). The concentration producing maximal inhibition of the 20 S/26 S proteasome complex was similar to that seen for the 20 S proteasome complex (> 10 μM), based on 50% maximal inhibition (Fig. 4*B*). Aggregated α-synuclein also inhibited the 20 S/26 S ubiquitin-independent proteasomal mixture. Based on an estimated molecular weight for aggregated α-synuclein of 160,000, we calculated that the IC_{50} of aggregated α-synuclein for the 20 S/26 S proteasome was 1 nM (Fig. 4*A*). The ability of aggregated α-synuclein to inhibit a mixture of the 26 S and 20 S proteasomes, but not the 20 S proteasome, suggests that aggregated

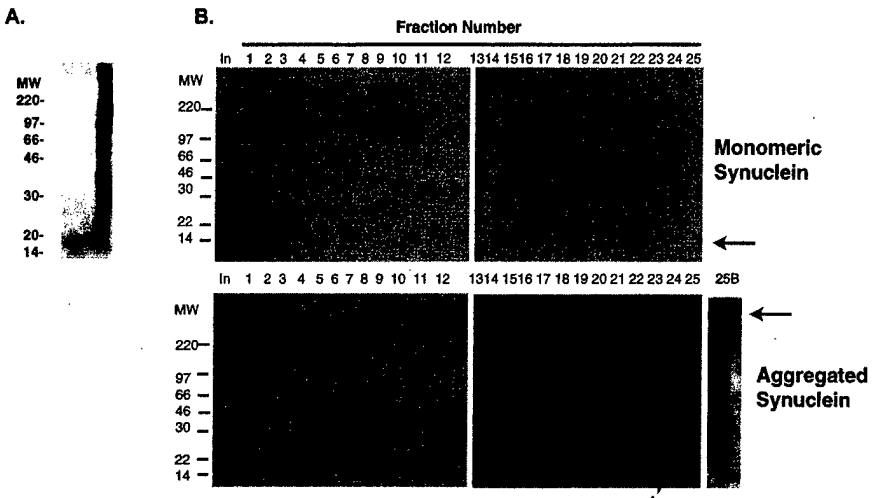


FIG. 2. **Analysis of monomeric and aggregated α-synuclein.** *A*, fresh α-synuclein (*lane 1*) migrated at about 16,000, which is consistent with a monomeric size. Aggregated α-synuclein (*lane 2*) exhibited both low and high molecular weight species following the process of immunoblotting, which involved heating in 2% SDS for 5 min, running on PAGE, and immunoblotting. This is a representative immunoblot from an experiment that had been repeated three times. *B*, samples of fresh (*top*) and aged (*bottom*) α-synuclein were fractionated on a 5–30% sucrose gradient, and each fraction was immunoblotted. An immunoblot of the non-fractionated starting material is shown in the first lane, *IN′*. The monomeric α-synuclein was present predominantly in the early fractions, suggesting a low molecular weight; the aged α-synuclein was present in the last fraction, suggesting a high molecular weight and little if any free monomeric α-synuclein. Because some SDS-sensitive aggregated α-synuclein appeared to be present in the aged Input sample, we overexposed the aged fractionated sample to determine whether it also contained any SDS-sensitive aged α-synuclein. A long exposure of fraction 25 (*lane 25B*) demonstrates the presence of 16,000 α-synuclein, suggesting that some of the aged, aggregated α-synuclein can be dissociated by SDS. This is a representative immunoblot from an experiment that had been repeated three times.

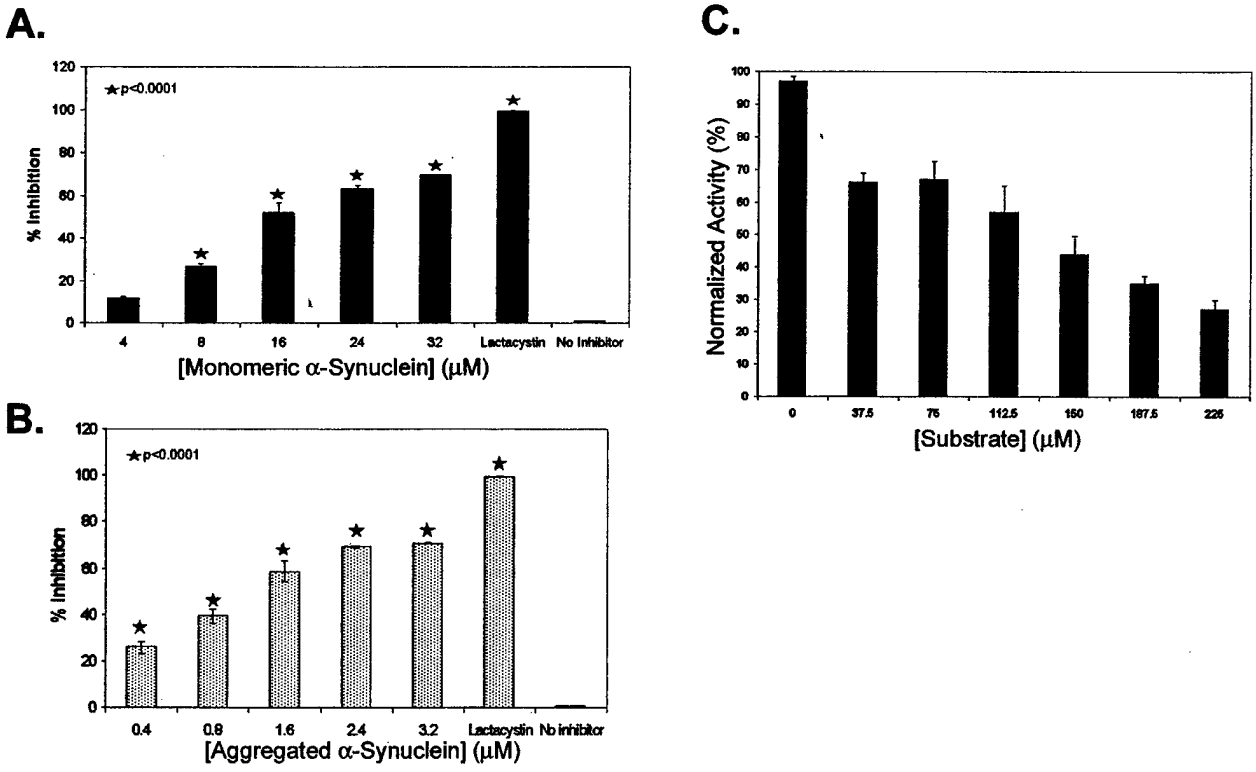


FIG. 3. **Effects of monomeric and aggregated α-synuclein on the 20 S proteasome.** *A*, inhibition of the 20 S proteasome by monomeric α-synuclein dose response. Five data points were used for each sample. *B*, inhibition of the 20 S proteasome by aggregated α-synuclein dose response. Five data points were used for each sample. *C*, substrate dependence of proteasomal inhibition by α-synuclein. Five data points were used for each sample.

α-synuclein selectively inhibits the 26 S proteasome.

Aggregated Synuclein, but Not Monomeric Synuclein, Inhibits Protein Degradation by the 26 S Proteasome—The greater ability of aggregated α-synuclein compared with monomeric α-synuclein in inhibiting 26 S ubiquitin-independent proteasomal activity raises the possibility that 26 S ubiquitin-dependent proteasomal function might also be selectively inhibited by aggregated α-synuclein. To investigate this, we examined ubiquitin-mediated degradation of a fusion protein made up of barnase and *E. coli* dihydrofolate reductase that had been fused with an N-terminal degradation tag (DHFR-U) (45). Prior studies show that degradation of ubiquitinated DHFR-U by reticulocyte lysates is mediated by the 26 S proteasome (45). We used this system to investigate how monomeric and aggregated α-synuclein affect ubiquitin-mediated proteasomal degradation. Degradation of ubiquitinated DHFR-U was examined

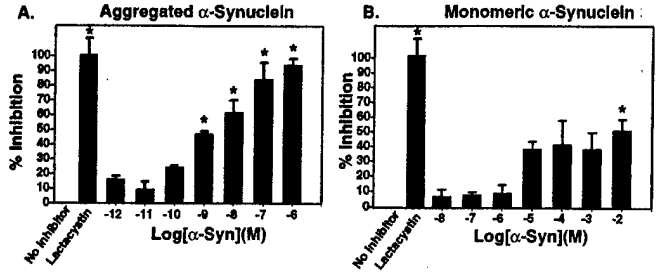


FIG. 4. **Inhibition of ubiquitin-independent proteasomal degradation by the 20 S/26 S proteasome with aggregated (*A*) and monomeric (*B*) α-synuclein using HEK 293 lysates.** The percent inhibition was normalized to the inhibition produced by the proteasomal inhibitor lactacystin (25 μM). *, $p < 0.002$ compared with no inhibitor.

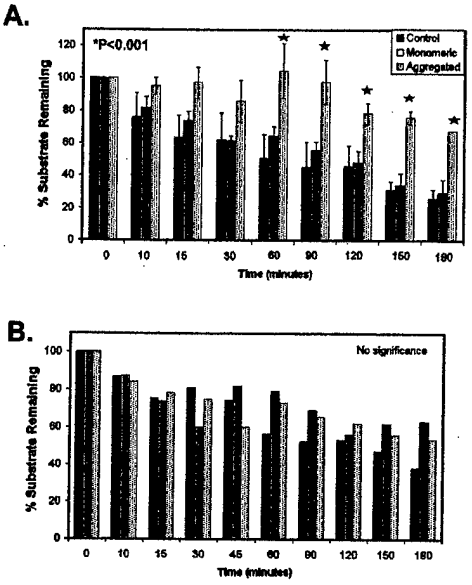


FIG. 5. **Inhibition of ubiquitin-dependent proteasomal degradation by aggregated α-synuclein.** *A*, aggregated α-synuclein (0.5 μM) inhibits ubiquitin-dependent degradation of DHFR-U by reticulocyte lysates by the 26 S proteasome, whereas monomeric α-synuclein (5 μM) does not inhibit degradation of DHFR-U by reticulocyte lysates by the 26 S proteasome. The overall analysis of variance was significant at $p < 0.001$. *Stars* show significance relative to DHFR-U in the absence of added α-synuclein. Each sample point was performed in triplicate. *B*, lack of inhibition of Clp1 by monomeric or aggregated α-synuclein. Degradation of DHFR-U was not significantly different between degradation of DHFR-U alone or in the presence of aggregated (0.5 μM) or monomeric (5 μM) α-synuclein. Each sample point was performed in triplicate.

in the presence of monomeric or aggregated α-synuclein (Fig. 5*A*). The half-life of DHFR-U was 125 min both under basal conditions and in the presence of 5 μM monomeric α-synuclein (Fig. 5*A*, *gray bars*). However, the half-life of DHFR-U greatly increased in the presence of 500 nM aggregated α-synuclein (Fig. 5*A*, *dotted bars*). No inhibition was seen with 50 nM aggregated α-synuclein (data not shown). These data indicate that 26 S ubiquitin-dependent proteasomal degradation is selectively inhibited by aggregated α-synuclein.

To determine whether inhibition of ubiquitin-dependent proteasomal degradation was specific to the 26 S proteasome, we also examined degradation of barnase that had been fused with a 65-amino acid N-terminal tag (DHFR-65) that allows the protein to be recognized and degraded by the bacterial proteasomal analog ClpAP (45). DHFR-65 was incubated with ClpAP alone or in the presence of 5 μM monomeric α-synuclein or in the presence of 500 nM aggregated α-synuclein, and the rate of degradation was monitored. Neither monomeric nor aggregated α-synuclein inhibited degradation of DHFR-65 by ClpAP (Fig. 5*B*). This indicates that proteasomal inhibition by aggregated α-synuclein is specific for the ubiquitin-dependent 26 S proteasomal system.

Native and Aggregated α-Synuclein Bind S6′—The ability of aggregated α-synuclein to inhibit degradation mediated by the 26 S proteasome could be explained by interaction between aggregated α-synuclein and a protein in the 19 S cap that is present in the 26 S proteasome but not the 20 S proteasome. Studies with rat α-synuclein suggest that α-synuclein binds the rodent 19 S proteasomal component S6′ (35). Based on this work, we investigated whether human α-synuclein interacts with S6′. His-tagged recombinant native or aggregated α-synuclein was incubated overnight with substantia nigra or cingulated cortex from normal human brain and then precipitated with nickel-agarose. The precipitates were immunoblotted with antibodies to S6′. A representative immunoblot with native α-synuclein is shown in Fig. 6*A*, and a pull-down with native or aggregated α-synuclein is shown in Fig. 6*B*. Both aggregated and monomeric α-synuclein associated with S6′. The term 'native' is used in this discussion because the overnight incubation of recombinant α-synuclein with the lysates appeared to promote formation of some recombinant α-synuclein dimer, in addition to the more abundant α-synuclein monomer (Fig. 6*B*, *lower panel*). Co-association of α-synuclein with S6′ was also observed by immunoprecipitating endogenous α-synuclein and immunoblotting for S6′ (Fig. 6*C*). To test the selectivity of the association, we examined whether α-synuclein binds other components of the 19 S proteasomal cap, such as Rpn12 and subunit 10b. Neither Rpn12 nor subunit 10b was observed to co-precipitate with α-synuclein (Fig. 6*D*, immunoblot for *10b* shown). It was not possible to test the association of S6′ with α-synuclein by immunoprecipitating S6′, because none of the antibodies to S6′ that we tested were successfully able to precipitate S6′ (data not shown). Together, these data suggest that both monomeric and aggregated α-synuclein bind S6′.

DISCUSSION

Proteasomal inhibition is known to be toxic to many cell types and is thought to contribute to the pathophysiology of neurodegenerative diseases (17, 18, 47). Our data demonstrate that overexpressing α-synuclein inhibits 20 and 26 S proteasomal activity. The relationship between overexpressed α-synuclein and the pathophysiology of PD, though, is unclear. Overexpressing α-synuclein in mammalian neurons does not lead to its spontaneous aggregation, except after delays of 6–12 months (48–51). Because protein aggregation is thought to play a critical role in the pathophysiology of neurodegenerative diseases and aggregation of α-synuclein appears to be important to the pathophysiology of PD, we sought to design experiments that would allow analysis of the actions of aggregated α-synuclein. To investigate whether aggregated α-synuclein interacts with the proteasome, we examined the behavior of α-synuclein that had been aggregated *in vitro*. We observed that aggregated α-synuclein inhibits both ubiquitin-dependent and -independent 26 S proteasomal activity. The IC_{50} of aggregated α-synuclein for ubiquitin-independent 26 S proteasomal activity was 1 nM, which was over 1000-fold higher than the IC_{50} for 20 S proteasomal activity. In contrast, monomeric α-synuclein inhibited 20 and 26 S proteasomal activity with an $IC_{50} > 10$ μM.

The high affinity of aggregated α-synuclein for inhibiting 26 S proteasomal activity could be explained by binding of aggregated α-synuclein to a protein in the 19 S cap, which is the proteasomal complex that binds to the 20 S proteasome and confers ubiquitin-dependence, as discussed below (3). Consist-

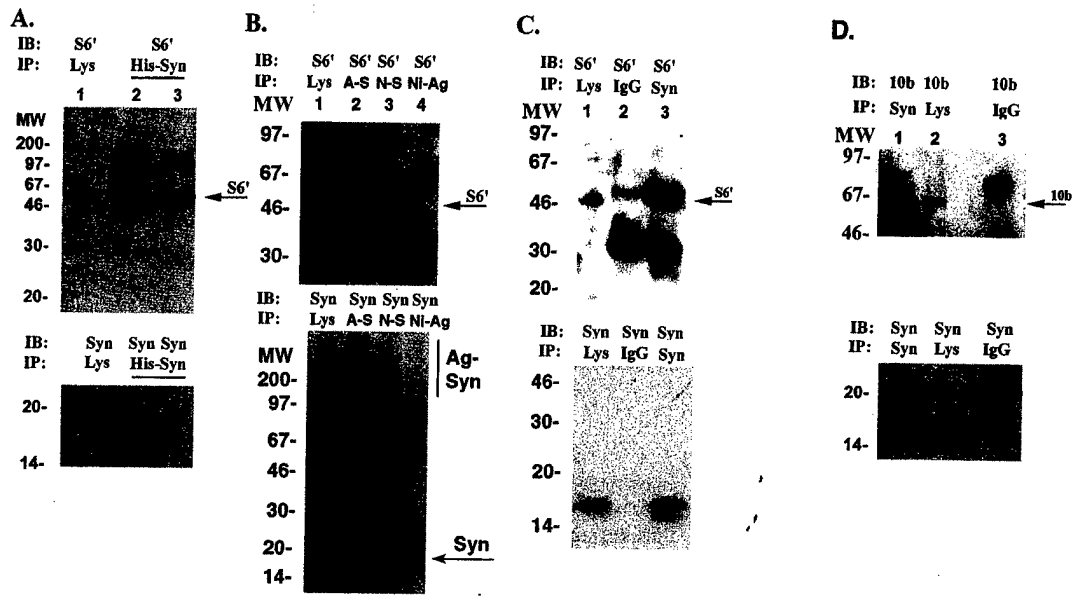


FIG. 6. **α-Synuclein binds S6′.** *A, upper panel,* immunoblot showing S6′ in brain lysate (*lane 1,* 10 μg) and precipitation of S6′ by monomeric His-tagged recombinant α-synuclein (*lanes 2* and *3*). *Lower panel,* reprobe of the same immunoblot with anti-α-synuclein antibody. The *arrow* points to the band corresponding to the S6′ protein. The α-synuclein band in lane 1 is lower than that in lanes 2 and 3 in the lower panel because the protein in lane 1 is endogenous α-synuclein, whereas the protein in lane 2 and 3 is His-tagged protein. *B, upper panel,* immunoblot showing S6′ in brain lysate (*lane 1,* 30 μg), precipitation of S6′ by aggregated (*lane 2, A-S*) or native His-tagged recombinant α-synuclein (*lane 3, N-S*), and lack of precipitation of S6′ using Ni-agarose pull-down without recombinant α-synuclein (*lane 4*). The *arrow* points to the band corresponding to the S6′ protein. *Lower panel,* reprobe of the same immunoblot with anti-α-synuclein antibody. The *arrow* points to monomeric α-synuclein, and the *bar* demonstrates the position of aggregated α-synuclein. The band at 36 kDa in lane 3 likely represents an α-synuclein dimer that might have been promoted by incubation of a large amount of recombinant α-synuclein with lysate but was not present in most other experiments (for example, see *panel C*). *C, upper panel,* immunoblot of S6′ showing the association of S6′ with α-synuclein following an immunoprecipitation of endogenous α-synuclein by anti-synuclein antibody. The *arrow* points to the band corresponding to the S6′ protein. *Lane 1* (*Lys*), crude brain lysates that had not been subject to immunoprecipitation. *Lane 2* (*IgG*), immunoprecipitation with nonspecific preimmune IgG antibody. *Lane 3* (*Syn*), immunoprecipitation with anti-α-synuclein antibody. *Lower panel,* reprobe with anti-α-synuclein antibody. *D,* no association was observed between α-synuclein and other proteasomal components, such as the 19 S subunit 10b. The *arrow* points to the band corresponding to the 10b protein, which is present in the lysates (*lane 2*) but not immunoprecipitated with α-synuclein (*lane 1*) or nonspecific IgG (*lane 3*). The abbreviations for this panel are the same as for panel B. All immunoblots in this figure are representative immunoblots from experiments that had been repeated at least three times.

ent with this hypothesis, we observed that α-synuclein binds to S6′, which is a subunit of the 19 S cap that was recently shown to bind polyubiquitinated proteins (3). Both aggregated and monomeric α-synuclein bind the S6′ protein. The interaction appears to be selective for S6′ because no association was observed with other 19 S proteasomal proteins, such as Rpn12 or subunit 10b.

Binding of α-synuclein to S6′ is consistent with prior publications. Ghee *et al.* (35) demonstrated that rat S6′ (also termed Tat binding protein-1, TBP1) binds α-synuclein using the yeast two-hybrid method. The association was confirmed by showing that an epitope-tagged S6′ could pull down α-synuclein following transfection of both proteins into HEK 293 cells. However, this study did not demonstrate interaction using the endogenous proteins and also did not investigate whether human α-synuclein binds to human S6′. In addition, Ii *et al.* (52) have documented the presence of proteasomal proteins in Lewy bodies, which supports our observation that aggregated α-synuclein binds S6′. The information presented in this study provides the functional relevance for these observations by showing that binding of α-synuclein to the proteasome inhibits proteasomal function.

The function of S6′ was recently identified and suggests a mechanism explaining why aggregated α-synuclein might inhibit the activity of the 26 S proteasome. The S6′ protein appears to function in the 19 S proteasomal cap as the docking protein for ubiquitin-conjugated proteins and is essential for binding of ubiquitin-conjugated proteins by the proteasome (3). Because aggregated α-synuclein is much larger than monomeric α-synuclein and often contains covalent cross-links, binding to S6′ might inhibit the function of the 19 S protein by competing with binding of other ubiquitin-conjugated proteins. Bound aggregated α-synuclein might occupy the unfolding proteins associated with proteasomal degradation, and the aggregate might also physically block the pore of the 19 S cap. This model provides an explanation for the ability of aggregated α-synuclein to interfere with both the ubiquitin-dependent and -independent 26 S proteasomal function.

Many other protein aggregates have been shown to be toxic to cells (18). Both aggregated cystic fibrosis transmembrane receptor and polyglutamine repeat exhibit toxicity that correlates with proteasomal inhibition (17, 18, 47). This study focuses attention on the interaction between S6′ and protein aggregates. Whether S6′ has a particular affinity for α-synuclein or is a general target for all protein aggregates remains to be determined. Inhibiting the ubiquitin-dependent proteasomal system (UPS) is known to be toxic, perhaps because it induces apoptosis (19). Inhibiting the UPS causes the accumulation of many toxic proteins, such as Pael-R, which was recently identified as a parkin substrate (53). Inhibiting the UPS is also known to cause the accumulation of protein aggregates in the endoplasmic reticulum (17, 47). Inhibiting the UPS could alter the regulation of cell cycle proteins (54). Reduced degradation of cell cycle proteins could account for the apparent abnormal activation of the cell cycle proteins observed in many neurodegenerative processes (55, 56).

Proteasomal inhibitors have recently been shown to induce degeneration of the dopaminergic neurons of the substantia nigra and induce α-synuclein aggregation (57). The tendency of α-synuclein to accumulate under conditions of proteasomal in-

hibition raises the possibility that the accumulation of aggregated α-synuclein adds to the proteasomal inhibition and increases the toxicity associated with proteasomal inhibition.

The discordance between the rapid kinetics of cell death associated with UPS inhibition in cell culture and the slow nature of degeneration in PD is notable. This discordance might be explained by the slow appearance of aggregated α-synuclein. α-Synuclein does not form aggregates under basal conditions when transiently overexpressed, but studies in transgenic mice show that overexpressing α-synuclein does lead to a delayed accumulation of aggregated α-synuclein (48–51). The slow rate of accumulation of aggregated α-synuclein could also lead to a correspondingly gradual inhibition of the UPS during the course of PD. Hence, progressive inhibition of the UPS by aggregated α-synuclein might be a gradual process in PD. Together these data suggest a model in which the gradual accumulation of aggregated α-synuclein progressively inhibits S6′ function, which leads to a gradual but progressive inhibition of the UPS and the progressive neurodegeneration that occurs in PD.

Acknowledgments—We thank John Hardy (National Institutes of Health) for providing the α-synuclein cDNA, Philipp Kahle (University of Munich) for useful comments, and Thomas Gallagher (Loyola University Medical Center) for insight into sucrose gradient methodology.

REFERENCES

1. Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998) *Mol. Cell. Biol.* **18,** 3149–3162
2. Voges, D., Zwickl, P., and Baumeister, W. (1999) *Annu. Rev. Biochem.* **68,** 1015–1068
3. Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (2002) *Nature* **416,** 763–767
4. Davies, K. J. (2001) *Biochimie (Paris)* **83,** 301–310
5. Shringarpure, R., Grune, T., and Davies, K. J. (2001) *Cell. Mol. Life Sci.* **58,** 1442–1450
6. Rechsteiner, M. (1991) *Cell* **66,** 615–618
7. Rechsteiner, M., Hoffman, L., and Dubiel, W. (1993) *J. Biol. Chem.* **268,** 6065–6068
8. Goldberg, A. L. (1992) *Eur. J. Biochem.* **203,** 9–23
9. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
10. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–847
11. Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998) *Cell* **92,** 367–380
12. Tanaka, K. (1998) *J. Biochem. (Tokyo)* **123,** 195–204
13. Tanaka, K. (1998) *Biochem. Biophys. Res. Commun.* **247,** 537–541
14. Satyal, S. H., Schmidt, E., Kitagawa, K., Sondheimer, N., Lindquist, S., Kramer, J. M., and Morimoto, R. I. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5750–5755
15. Paulson, H. L., Perez, M. K., Trottier, Y., Trojanowski, J. Q., Subramony, S. H., Das, S. S., Vig, P., Mandel, J. L., Fischbeck, K. H., and Pittman, R. N. (1997) *Neuron* **19,** 333–344
16. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S. U., Bredesen, D. E., Tufaro, F., and Hayden, M. R. (1998) *Nat. Genet.* **18,** 150–154
17. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* **292,** 1552–1555
18. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) *Nature* **416,** 507–511
19. Jesenberger, V., and Jentsch, S. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 112–121
20. Spillantini, M., Schmidt, M., Lee, VM-Y, Trojanowski, J., Jakes, R., and Goedert, M. (1997) *Nature* **388,** 839–840
21. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6469–6473
22. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) *Science* **276,** 2045–2047
23. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J., Schols, L., and Riess, O. (1998) *Nature Gen.* **18,** 106–108
24. Conway, K., Harper, J., and Lansbury, P. (1998) *Nature Med.* **4,** 1318–1320
25. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 571–576
26. Wood, S., Wypych, J., Steavenson, S., Louis, J., Citron, M., and Biere, A. (1999) *J. Biol. Chem.* **274,** 19509–19512
27. Biere, A. L., Wood, S. J., Wypych, J., Steavenson, S., Jiang, Y., Anafi, D., Jacobsen, F. W., Jarosinski, M. A., Wu, G. M., Louis, J. C., Martin, F., Narhi, L. O., and Citron, M. (2000) *J. Biol. Chem.* **275,** 34574–34579
28. Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Alexander, P., Choi, P., Palacino, J., Hardy, J., and Wolozin, B. (1999) *J. Neurosci.* **19,** 5782–5791
29. Jenco, J., Rawlingson, A., Daniels, B., and Morris, A. (1998) *Biochemistry* **37,** 4901–4909
30. Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000) *J. Biol. Chem.*
31. Engelender, S., Kaminsky, Z., Guo, X., Sharp, A., Amaravi, R., Kleiderlein, J., Margolis, R., Troncoso, J., Lanahan, A., Worley, P., Dawson, V., Dawson, T., and Ross, C. (1999) *Nature Gen.* **22,** 110–114
32. Sharon, R., Goldberg, M. S., Bar-Josef, I., Betensky, R. A., Shen, J., and Selkoe, D. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9110–9115
33. Choi, P., Golts, N., Snyder, H., Petrucelli, L., Chong, M., Hardy, J., Sparkman, D., Cochran, E., Lee, J., and Wolozin, B. (2001) *NeuroReport* **12,** 2839–2844
34. Lee, F. J., Liu, F., Pristupa, Z. B., and Niznik, H. B. (2001) *FASEB J.* **15,** 916–926
35. Ghee, M., Fournier, A., and Mallet, J. (2000) *J. Neurochem.* **75,** 2221–2224
36. Wakabayashi, K., Engelender, S., Yoshimoto, M., Tsuji, S., Ross, C. A., and Takahashi, H. (2000) *Ann. Neurol.* **47,** 521–523
37. Schlossmacher, M. G., Frosch, M. P., Gai, W. P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharon, R., Hattori, N., Langston, J. W., Mizuno, Y., Hyman, B. T., Selkoe, D. J., and Kosik, K. S. (2002) *Am. J. Pathol.* **160,** 1655–1667
38. Souza, J. M., Giasson, B. I., Lee, V. M., and Ischiropoulos, H. (2000) *FEBS Lett.* **474,** 116–119
39. Tanaka, Y., Engelender, S., Igarashi, S., Rao, R. K., Wanner, T., Tanzi, R. E., Sawa, A., Dawson, V. L., Dawson, T. M., and Ross, C. A. (2001) *Hum. Mol. Genet.* **10,** 919–926
40. Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J., Farrer, M., and Wolozin, B. (2000) *J. Neurosci.* **20,** 6048–6054
41. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., and Wolozin, B. (2002) *J. Biol. Chem.* **277,** 16116–16123
42. Lee, H. J., Choi, C., and Lee, S. J. (2001) *J. Biol. Chem.* **25,** 25
43. Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000) *Nat. Neurosci.* **3,** 1301–1306
44. Uversky, V. N., Li, J., and Fink, A. L. (2001) *FEBS Lett.* **500,** 105–108
45. Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., and Matouschek, A. (2001) *Mol. Cell* **7,** 627–637
46. Matouschek, A., Azem, A., Ratliff, K., Glick, B. S., Schmid, K., and Schatz, G. (1997) *EMBO J.* **16,** 6727–6736
47. Johnston, J., Ward, C., and Kopito, R. (1998) *J. Cell Biol.* **143,** 1883–1898
48. Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A., and Price, D. L. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**
49. Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., and Lee, V. M. (2002) *Neuron* **34,** 521–533
50. Kahle, P. J., Neumann, M., Ozmen, L., Muller, V., Odoy, S., Okamoto, N., Jacobsen, H., Iwatsubo, T., Trojanowski, J. Q., Takahashi, H., Wakabayashi, K., Bogdanovic, N., Riederer, P., Kretzschmar, H. A., and Haass, C. (2001) *Am. J. Pathol.* **159,** 2215–2225
51. Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000) *Science* **287,** 1265–1269
52. Ii, K., Ito, H., Tanaka, K., and Hirano, A. (1997) *J. Neuropathol. Exp. Neurol.* **56,** 125–131
53. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, Y. (2001) *Cell* **105,** 891–902
54. Yew, P. R. (2001) *J. Cell. Physiol.* **187,** 1–10
55. Husseman, J. W., Nochlin, D., and Vincent, I. (2000) *Neurobiol. Aging* **21,** 815–828
56. Chang, B. D., Watanabe, K., Broude, E. V., Fang, J., Poole, J. C., Kalinichenko, T. V., and Roninson, I. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4291–4296
57. McNaught, K. S., Bjorklund, L. M., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C. W. (2002) *Neuroreport* **13,** 1437–1441

Appendix 2

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Molecular Brain Research x (2003) xxx–xxx
MOLECULAR BRAIN RESEARCH
www.elsevier.com/locate/molbrainres

Research report

SEPT5_v2 is a parkin-binding protein

P. Choi[a,1], H. Snyder[a,1], L. Petrucelli[b], C. Theisler[a], M. Chong[a], Y. Zhang[c], K. Lim[c], K.K.K. Chung[c], K. Kehoe[c], L. D'Adamio[d], J.M. Lee[a], E. Cochran[e], R. Bowser[f], T.M. Dawson[c], B. Wolozin[a,*]

[a]Department of Pharmacology, Loyola University Medical Center, Bldg. 102/3634, 2160 S. 1st Ave., Maywood, IL 60153, USA
[b]Department of Pharmacology, Mayo Clinic, Jacksonville, FL, USA
[c]Department of Neurology and Neuroscience, Johns Hopkins Medical Center, Baltimore, MD, USA
[d]Department of Microbiology and Immunology, Albert Einstein College of Medicine, The Bronx, NY, USA
[e]Department of Pathology, St. Lukes-Rush-Presbyterian Medical Center, Chicago, IL, USA
[f]Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA

Accepted 24 June 2003

Abstract

Mutations in parkin are associated with various inherited forms of Parkinson's disease (PD). Parkin is a ubiquitin ligase enzyme that catalyzes the covalent attachment of ubiquitin moieties onto substrate proteins destined for proteasomal degradation. The substrates of parkin-mediated ubiquitination have yet to be completely identified. Using a yeast two-hybrid screen, we isolated the septin, human SEPT5_v2 (also known as cell division control-related protein 2), as a putative parkin-binding protein. SEPT5_v2 is highly homologous to another septin, SEPT5, which was recently identified as a target for parkin-mediated ubiquitination. SEPT5_v2 binds to parkin at the amino terminus and in the ring finger domains. Several lines of evidence have validated the putative link between parkin and SEPT5_v2. Parkin co-precipitates with SEPT5_v2 from human substantia nigra lysates. Parkin ubiquitinates SEPT5_v2 in vitro, and both SEPT5_v1 and SEPT5_v2 accumulate in brains of patients with ARJP, suggesting that parkin is essential for the normal metabolism of these proteins. These findings suggest that an important relationship exists between parkin and septins.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder [21]. It is characterized by a classic group of symptoms: rigidity, resting state tremor, bradykinesia, and postural instability. Death of dopaminergic neurons in the substantia nigra with resultant severe dopamine depletion in the neostriatum is believed to underlie the motor symptoms of PD [21]. The cause of this neuronal degeneration is unknown. Recently, several genes have been identified to be associated with familial parkinsonism: α-synuclein, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), DJ-1, Nurr1 and parkin [3,10,18,20,22,25,29]. Mutations in α-synuclein (A53T and A30P) are associated with rare cases of autosomal dominant parkinsonism [20,29]. An I93M missense mutation in the ubiquitin C-terminal hydrolase, UCH-L1, a thiol protease deubiquitinating enzyme, has been identified in a German family with a history of familial PD [23]. Mutations in DJ-1 are associated with an early onset, autosomal recessive form of PD, while parkin is associated with autosomal recessive juvenile parkinsonism (ARJP) [3,18]. Mutations in Nurr1, an orphan nuclear receptor that is essential for the development of dopamine neurons, are associated with familial PD [22]. The most common mutations associated with Parkinsonism are mutations in *parkin*, including deletion and point mutations, which occur in many families affected by ARJP. The association of mutations in *parkin* with ARJP implicates *parkin* mutations in the etiology of familial parkinsonism [1,24,25]. In the most comprehensive genetic study to date

Abbreviations: ARJP, autosomal recessive Juvenile Parkinsonism; PD, Parkinson's disease; SEPT5, cdc-rel1, cell division control-related protein 1; SEPT5_v2, SEPT5_v2, cell division control-related protein 2; UbcH, ubiquitin conjugating enzyme

*Corresponding author. Tel.: +1-708-216-6195; fax: +1-708-216-6596.
E-mail address: bwolozi@lumc.edu (B. Wolozin).
[1]Co-equal authors.

0169-328X/03/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0169-328X(03)00318-8

by Lücking et al., 36 of 73 (49%) families with histories of early onset PD all had mutations in *parkin* that segregated with the disease [25]. In addition, mutations in parkin have also been recently linked to forms of adult-onset parkinsonism clinically indistinguishable from sporadic, non-inherited PD [8,19]. Thus, mutations in *parkin* are by far the most prevalent cause of inherited PD discovered to date.

The parkin structure contains a C-terminal RING-IBR-RING construct and an N-terminal region with homology to ubiquitin [18,31]. Recently, parkin was shown to possess ubiquitin ligase (E3) activity that catalyzes the covalent attachment of ubiquitin onto proteins targeted for degradation by the 26S proteasome [14,31,35]. Ubiquitination of proteins is thought to occur by action of a complex that includes E3 ligases, such as parkin, and ubiquitin conjugating enzymes (termed either UbcH or E2). Nine human UbcH enzymes have been identified to date, and parkin has been shown to associate with UbcH7 and UbcH8, via the RING-IBR-RING domain of parkin. Intense research has focused on identifying proteins that bind parkin and are ubiquitinated by parkin. Several parkin-binding proteins have been identified to date: cell division control-related protein 1, α-synuclein, Pael-R, Synphilin-1 and CHIP [6,13,14,32,36]. SEPT5 is a member of the septin family of proteins, which have roles in exocytosis and cell division [2]. Other septins include Nedd5, H5, Diff6, and SEPT5_v2 [2,17]. Wild-type parkin, but not mutant or truncated forms of parkin, increases SEPT5_v1 turnover in vitro, suggesting a central role of parkin in SEPT5_v1 regulation [36]. Parkin has also been shown to control the turnover of SEPT5_v1 and the Pael receptor (Pael-R) [14,36]. The interaction of parkin with CHIP suggests that it operates in the endoplasmic reticulum as part of the unfolded protein response [13,15]. We now report the identification of another parkin-binding protein, SEPT5_v2. Parkin ubiquitinates SEPT5_v2, and SEPT5_v2 steady state levels are increased in brains of patients with ARJP or in cells lacking parkin.

2. Materials and methods

2.1. Human brain samples

Post-mortem brain tissues were obtained from the Loyola University Chicago Brain Bank and from the Rush University Brain Bank. Paraffin-embedded tissues used for immunohistochemical analyses were treated with 10% buffered formalin. Human substantia nigra sections from PD cases and age-matched, neurologically normal controls were examined in this study. The ages and post-mortem interval of all the brains used are shown in Table 1. The mean ages and PMI of the control brains were 73.5±5.7 years and 7.6±1.8 h, while the means ages and post-mortem interval of the PD brains were 68.4±1.4 years and 7.0±1.1 h.

Table 1
Brain samples

Case	PMI	Age	Sex	Diagnosis
1	5	64	M	Control
2	10.5	64	F	Control
3	11	79	F	Control
4	4	87	M	Control
5	5	64	F	PD
6	6	68	M	PD
7	4.5	72	M	PD
8	10	67	F	PD
9	9.5	71	M	PD
10	10	65 (17)		ARJP exon 3 deletion
11	18	62 (24)		ARJP exon 4 deletion
12	9	52 (14)		ARJP exon 4 deletion
13	10	68 (33)		ARJP exon 3 deletion

Paraffin-embedded brain sections were deparaffinized by incubation at 65 °C for 45 min. Sections were subsequently treated with three changes in xylene, 10 min each, then 3 min each in 100% ethanol, 95% ethanol, and 70% ethanol. Sections were then washed for 5 min in ddH_2O, placed in fresh ddH_2O, and autoclaved for 15 min at 121 °C. After slides cooled to room temperature, they were washed in 0.05 M PBS, pH 7.4, for 20 min, blocked in 10% FBS/PBS for 20 min, and incubated at 4 °C overnight in primary antibodies. This was followed by three 10-min washes in PBS, incubation with Cy2- and Cy3-tagged fluorescent antibodies (Jackson Immuno.) for 3 h at room temperature, and three 10-min washes in PBS. Slides were then dehydrated through ethanol, mounted with Depex, and cover-slipped.

2.2. Immunoblotting

For immunoblot analyses, protein gel loading buffer was added to 30 μg of total protein lysate per lane and resolved by 12% polyacrylamide gel electrophoresis. Protein bands were transferred to nitrocellulose matrix (Gibco/BRL) at 4 °C, 250 mA, for 10 h. Following blocking in 5% milk/tris-buffered saline plus 0.01% triton X-100 for 1 h at room temperature, blots were incubated with primary antibody overnight at 4 °C. Blots were then washed three times, 10 min each in tris-buffered saline plus 0.01% triton X-100, and incubated in biotinylated secondary antibody for 3 h at room temperature, washed again, and developed by chemiluminescence (DuPont).

2.3. Antibodies

Park-1 polyclonal antibody (Southwest Immunology) was raised in goat, against an immunogen that corresponds to amino acids 83–97 of the N-terminus of the human parkin protein. Park-2 polyclonal antibody (Chemicon) was raised in rabbit, against an immunogen that corre-

sponds to amino acids 305–323 of the human parkin protein. Park-3 polyclonal antibody was also raised in rabbit, against an immunogen corresponding to amino acids 391–405 of the parkin protein. Park-4 polyclonal antibody was raised against an epitope around amino acid 400 of parkin (Cell Signaling, no epitope sequence supplied). SP20 is a monoclonal antibody that recognizes SEPT5. Anti-α-actin monoclonal antibody (ICN) was used as an internal loading control in Western blot analyses. The anti-c-Myc antibody 9E10 was from Roche. Dilutions were done in tris-buffered saline plus 0.01% triton X-100+5% bovine serum albumin (Sigma). Dilutions: Park-1—1:300 for immunohistochemistry, and 1:5000 for immunoblotting; Park-2—1:1000 for immunohistochemistry; Park-3—1:500 for immunoblotting, and 1:200 for immunoprecipitation; Park-4—1:1500 for immunoblotting; SP20—1:10 for immunohistochemistry, and 1:100 for immunoblotting; anti-α-actin—1:2000 for immunoblotting; 9E10—1:1000 for immunoblotting, 1:200 for immunoprecipitation.

2.4. Plasmids

Wild-type and mutant parkin were cloned into pcDNA3 at the EcoRI/XbaI sites. Myc-parkin was generated by adding a myc epitope by PCR, and inserted into pcDNA3 at the EcoRI/NotI sites. SEPT5_v2a and myc-SEPT5_v2a were cloned into pcDNA3 at the XhoI/NotI sites. For production of recombinant SEPT5_v2a, the cDNA was also cloned into the proEX vector at the BamHI/SpeI sites, and purified with nickel agarose chromatography. UbcH7 and 8 were cloned into the pET3a vector. HA-Ubiquitin was obtained from Cecile Pickart (Johns Hopkins, Baltimore, MD, USA) and was cloned into the pMT123 vector. The parkin deletion constructs were in pRK5 and designed as described previously [36].

2.5. Cell culture

BE-M17 human neuroblastoma cells were maintained in Optimem (Gibco/BRL)+10% fetal bovine serum. SH-SY5Y human neuroblastoma cells were maintained in a 1:1 mixture of Ham's F12:EMEM (Gibco/BRL)+10% fetal bovine serum. Transfections were performed with Fugene (Roche) with 1 μg DNA+6 μl Fugene per ml in Optimem.

2.6. Immunoprecipitation

For immunoprecipitation experiments, approximately 2×10^6 cells were plated into sterile 10-cm Falcon dishes and grown to ≈80% confluence. Cells were harvested by trituration followed by low-speed centrifugation. Pellets were resuspended in lysis solution consisting of ice-cold 1% Triton-X in Tris-buffered saline, pH 7.4, with protease inhibitors (Sigma) and dismembranated via sonication. Protein concentration was determined by the BCA assay (Pierce). 500 μg of protein from each sample was diluted to a final volume of 1 ml in lysis solution. 25 μl protein A (Sigma) was added to pre-clear non-specific binding proteins, 1 h, 4 °C. Samples were centrifuged and supernatants were transferred to new sterile microcentrifuge tubes. 4 μg of primary antibody were added to each sample, followed by a 3-h shaking incubation at 4 °C. 30 μl protein A were then added to each sample and binding to immune complexes was performed by 2 h incubation at 4 °C with gentle agitation. Negative control consisted of 4 μg non-specific IgG plus control brain lysate and protein A. Samples were washed/centrifuged four times in ice-cold lysis solution. After final washing and centrifugation, samples were resuspended in protein loading buffer, heat denatured at 90 °C for 5 min, centrifuged again, and resolved by 12% polyacrylamide gel electrophoresis, 85 V, 90 min. Immunoblots were performed as detailed above.

2.7. Yeast two-hybrid

Assays were performed according to the manufacturer's protocol, using the Matchmaker (Clontech) LexA system. The cDNA library used for screening bait/prey interactions was derived from human brain, and consisted of 3.5×10^6 independent clones. We performed an interaction trap assay using parkin as the bait. Full-length parkin (465 amino acids; bait A), the amino-terminal 133 amino acids (bait B), the carboxy-terminal 332 amino acids (bait C), and the amino-terminal 257 amino acids (bait D) were cloned into plasmid pEG202 in-frame with the LexA DNA-binding domain. Two different reporter genes were used in the LexA system: one was a yeast Leu2 derivative and the other was the bacterial lacZ gene that encodes β-galactosidase and offers a quantitative method of measuring interactions. The LEU2 reporter is stably incorporated into the yeast strain, and has its normal regulatory sequences replaced by six LexA operator sequences. The lacZ reporter was introduced into the yeast strain on a plasmid (pSH18-34) and is also regulated by multiple upstream LexA operators.

2.8. In vitro ubiquitination

Constructs coding for myc-Parkin or myc-SEPT5_v2a were transfected into 293 cells, and immunoprecipitated from 4 mg of lysates with 4 μl 9E10 antibody and 50 μl of agarose coupled protein G. 7 μl of each immunoprecipitate was mixed with 100 ng E1 (Sigma), 200 ng Ubch7 (Affinity), and 5 μg ubiquitin (Sigma) in 50 μl buffer (50 mM Tris HCl pH 7.5, 10 mM DTT, 2 mM $MgCl_2$ and 4 mM ATP). The mixture was incubated for 1 h and then immunoblotted.

3. Results

3.1. Parkin binds to SEPT5_v2

We utilized the LexA yeast two-hybrid system to screen a human brain cDNA library for binding partners of parkin. We isolated positive clones coding for the septin, SEPT5_v2, in both splice variants, rel2a and rel2b (Fig. 1A, SEPT5_v2A shown). These clones collectively encoded sequences at the N-terminus of rel2a from amino acid 1 to 304, and the N-terminus of rel2b from amino acid 1 to 295, in multiple, in-frame overlapping prey cDNA hybrids, indicating that these amino acids mediate the parkin–SEPT5_v2 association. The amino terminus of SEPT5_v2 shows minimal homology to other septins, including SEPT5_v1 (Fig. 1B). SEPT5_v2a and b are splice

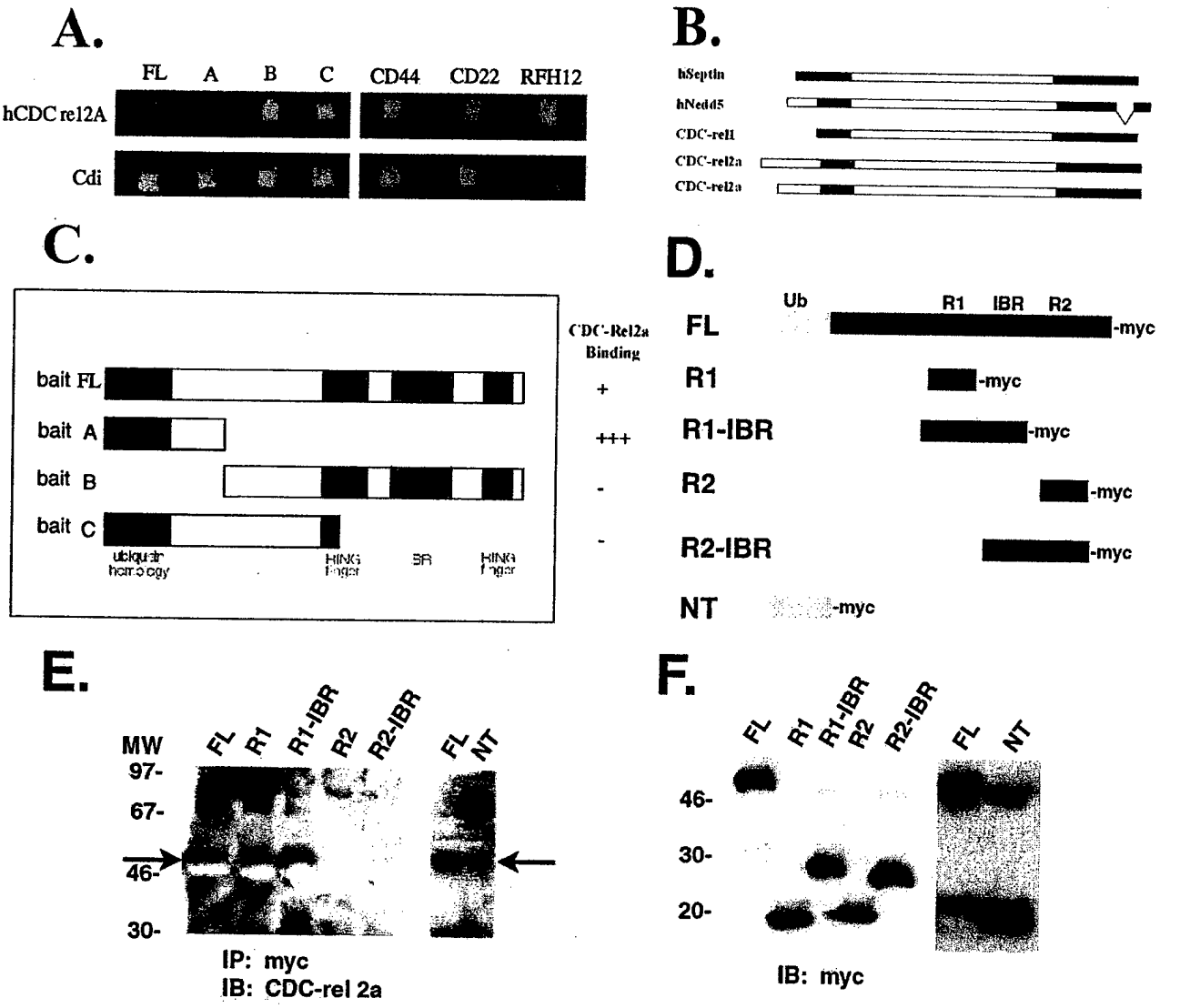


Fig. 1. (A) Yeast mating assay showing interaction between parkin and SEPT5_v2a. Blue represents a positive interaction. CDI is a control that should only interact with the RFH12 protein, thus demonstrating specificity for the assay. FL, full length parkin. (A, B, C) Parkin constructs shown below. CD44, CD22, and RFH12 are constructs that are not expected to interact with parkin, and act as negative controls. (B) Structure of septins. The structures of SEPT5_v2a, SEPT5_v2b, and SEPT5, are shown in relation to some other well-known septins, such as Septin, and Nedd5. Homologous regions are represented by the same color. Adapted from Kartmann and Roth [16]. (C) Structure of yeast parkin-binding constructs, and amount of binding to SEPT5_v2 on the right. (D) Structure of mammalian parkin deletion constructs. Myc-tagged constructs containing full-length parkin (FL), the R1 RING finger domain (R1), the R1 RING finger domain plus the 'In Between RING' finger domain (R1-IBR), the R2 RING finger domain (R2), the R2 RING finger domain plus the 'In Between RING' finger domain (R2-IBR), and the N-terminal ubiquitin homology domain (NT). (E) Co-immunoprecipitation of SEPT5_v2a with myc-tagged parkin deletion constructs. Left panel: SEPT5_v2a (arrow) co-immunoprecipitated with full-length parkin, the R1 RING finger domain and the R1 RING finger domain plus the 'In Between RING' finger domain (R1-IBR). Right panel: SEPT5_v2a (arrow) co-immunoprecipitated with full-length parkin (FL), the N-terminal ubiquitin domain (NT). (F) Immunoblots validating expression of the parkin deletion constructs in the HEK 293 cell lysates used for the immunoprecipitation in (E).

variants, with SEPT5_v2b lacking the first 19 amino acids of SEPT5_v2a (Fig. 1B). Interestingly, in the case of SEPT5, it is the carboxy terminal of parkin that associates with the carboxy terminal of SEPT5_v1 [36]. All further experiments were performed using the LexA yeast two-hybrid system and SEPT5_v2a as the interacting protein.

To understand better the nature of the interaction between parkin and SEPT5_v2, we generated a construct containing full-length parkin, and constructs of parkin truncated at either the amino or carboxy terminal (Fig. 1C). Yeast two-hybrid mating assays were subsequently performed to map the area of parkin responsible for SEPT5_v2a binding. SEPT5_v2a bound to full-length parkin and an amino terminal construct, but not a construct lacking the amino terminus (Fig. 1A and C). This suggests that the ubiquitin homology portion of parkin interacts with SEPT5_v2. Interestingly, no binding was seen to construct C that contained the amino two-thirds of parkin, possibly because of the altered conformation of this construct or inhibition of binding by downstream domains of parkin (Fig. 1C).

3.2. SEPT5_v2a binds to parkin at two sites in mammalian cells

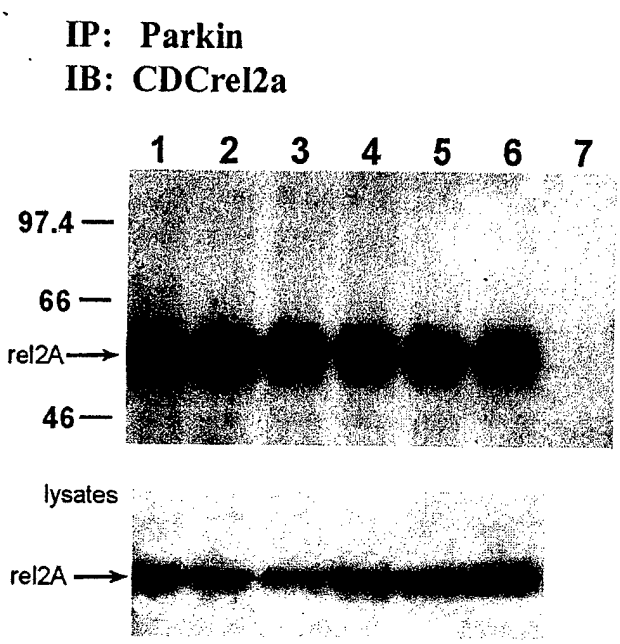
Previous studies with SEPT5, a close homologue of SEPT5_v2a, showed binding to the carboxy region of parkin [36]. Because of the close homology between the two proteins, we examined the association of SEPT5_v2a with domains of parkin previously shown to bind SEPT5_v1, including the RING-IBR domains (Fig. 1C). Based on the yeast two-hybrid data suggesting binding of SEPT5_v2a to a domain near the amino terminus of parkin, we also designed a construct containing only the amino-terminal ubiquitin homology domain, and examined binding of SEPT5_v2a to this domain (Fig. 1C). HEK 293 cells were co-transfected with constructs coding for SEPT5_v2a and myc-tagged parkin deletion constructs corresponding to the amino or carboxy domains of parkin (Fig. 1D and E) [36]. We observed that SEPT5_v2a bound to full-length parkin, as well as parkin deletion constructs containing either the amino domain of parkin or the R1 RING finger (Fig. 1D and E). Binding of SEPT5_v2a to the R1 RING finger domain is consistent with prior studies showing that SEPT5_v1 binds to the R2 RING finger domain [36]. In addition, we observed that SEPT5_v2a binds to the amino domain of parkin, which contains the ubiquitin homology domain. Binding of SEPT5_v2a to the amino region of parkin concurs with the yeast two-hybrid studies, which also showed binding of this region. This indicates that both SEPT5_v1 and SEPT5_v2a associate with the RING finger domains, with SEPT5_v1 binding to the R2 domain and SEPT5_v2a binding to the R1 domain [36].

Although binding to the amino domain and the R1-IBR-R2 domain of parkin show binding to SEPT5_v2 in both the yeast two-hybrid assay and in mammalian cell immunoprecipitation assay, the two assays differ quantitatively in the amount of binding observed (Fig. 1C and E). One reason for the difference might result from the use of different parkin domains in the yeast two-hybrid study and the immunoprecipitation study. One discrepancy lies in the parkin R1-IBR-R2 domain. A construct containing the parkin R1-IBR-R2 domain showed no association with SEPT5_v2a in the yeast two-hybrid study, while a construct containing only the R1-IBR domain showed strong binding in the immunoprecipitation assay (Fig. 1C and E). The absence of an association in the yeast two-hybrid assay might derive from inhibition of SEPT5_v2 binding to parkin by the R2 domain when it is present. This hypothesis could explain why the immunoprecipitation assay showed no binding to a construct containing only the IBR-R2 domain and weak binding to full-length parkin (Fig. 1C and E). Binding to the parkin amino domain also showed a quantitative difference between the yeast two-hybrid assay and the immunoprecipitation assay. This difference could be due to differences in the size of the two constructs (Fig. 1D and F). The parkin amino domain construct used for the yeast two-hybrid studies was longer than that used for the immunoprecipitation studies (Fig. 1D and F). Binding of SEPT5_v2 close to the junction region between the ubiquitin homology domain and the rest of parkin could render it sensitive to interference from the anti-myc antibody used for the immunoprecipitation assay (Fig. 1C and E). Conversely, the increased length of the amino domain yeast two-hybrid construct might prevent interference with SEPT5_v2 binding.

3.3. SEPT5_v2a binds to parkin in human brain lysates

To validate our yeast two-hybrid analysis results, we investigated whether parkin interacts with SEPT5_v2 in human brain. SEPT5_v2a co-precipitated with parkin from human substantia nigra lysates (Fig. 2). Sepharose-coupled anti-parkin antibody P391 was used to immunoprecipitate parkin, and the immunoprecipitate was then immunoblotted with anti-SEPT5_v2a (Fig. 2, upper panel). As a negative control, the lysates were immunoprecipitated with sepharose coupled to non-specific IgG (Fig. 2, upper panel, lane 7). Nigral lysates from both control (Fig. 2, lanes 1–3) and PD (Fig. 2, lanes 4–6) cases were used in the immunoprecipitations to examine whether disease-associated differences in parkin-SEPT5_v2 binding exist. Immunoprecipitation revealed a single band migrating at the expected molecular weight of 52 kDa (Fig. 2). Although significant enrichment of SEPT5_v2a was observed by immunoprecipitation of parkin, indicative of high-affinity binding, there was no significant difference in the amount of co-precipitated rel2a between PD and control brains (Fig. 2). The amount of SEPT5_v2a in the lysates was also immunoblotted (Fig. 2, lower panel). The PD nigral lysates showed a trend toward greater SEPT5_v2a levels in PD than control lysates, but this difference did not reach

416



417

418 Fig. 2. Co-precipitation of SEPT5_v2a along with parkin from substantia
419 nigra lysates. Sepharose-coupled anti-parkin antibody P391 was used to
420 immunoprecipitate parkin. Immunoblotting the precipitate with the anti-
421 SEPT5_v2a antibody revealed one band at 52 kDa, which is the expected
422 molecular weight of SEPT5_v2a. The top panel shows the immuno-
423 precipitate, while the bottom panel shows an immunoblot of the lysates
424 with anti-SEPT5_v2a. Lanes 1–3 correspond to substantia nigra from
425 three different control donors. Lanes 4–6 correspond to substantia nigra
426 from three different PD donors. Lane 7 is an immunoprecipitation using
427 non-specific rabbit IgG.

428 statistical significance ($P<0.1$, $N=9$ PD, 10 Control).
429 Immunocytochemical experiments failed to detect any
430 SEPT5_v2a in Lewy bodies (data not shown). The co-
431 immunoprecipitation of parkin and SEPT5_v2a indicates
432 that parkin associates with SEPT5_v2a in human brain
433 tissue, and validates the yeast two-hybrid results.

434 *3.4. Parkin levels affect levels of SEPT5_v2a*

435 The association of SEPT5_v2a with parkin raises the
436 possibility that the degradation of SEPT5_v2a is mediated
437 by parkin. To investigate this, HEK 293 cells were
438 transfected with plasmids coding for sense or anti-sense
439 parkin plus SEPT5_v2a, HA-tagged ubiquitin and UbcH8.
440 Three days following transfection, the cells were incubated
441 with MG132 for 4 h to block proteasomal degradation. The
442 cells were lysed, and centrifuged to separate soluble and
443 insoluble material. Levels of SEPT5_v2a in both the pellet
444 and supernatant were observed to vary with parkin levels.
445 Cell pellets were immunoblotted with antibodies against
446 HA (identifying ubiquitin), SEPT5_v2a, parkin or actin
447 (Fig. 3A and B). Parkin expression was high in cells
448 transfected with parkin cDNA and low in cells transfected
449 with parkin anti-sense cDNA (Fig. 3A, bottom left panel).
450 HA-ubiquitin immunoreactivity paralleled parkin expres-
451 sion (Fig. 3A, top left panel). The level of SEPT5_v2a was
452 inversely related to parkin expression, and was low in cells
453 transfected with parkin cDNA and high in cells transfected
454 with parkin anti-sense cDNA (Fig. 3B, left panel). Finally,
455 immunoblotting with anti-actin antibody showed that
456 protein expression was similar in each lane (Fig. 3B, right
457 panel).

458 *3.5. Parkin ubiquitinates SEPT5_v2a*

459 Next we examined whether SEPT5_v2a was ubiquiti-
460 nated by parkin. To determine whether parkin was able to
461 ubiquitinate SEPT5_v2a, we used an in vitro ubiquitination
462 assay, similar to that used for the analysis of ubiquitination
463 of Pael-R [14]. For the in vitro ubiquitination assay, HEK
464 293 cells were transfected cells with either myc-SEP-
465 T5_v2a or myc-parkin. After 48 h, the cells were harvested,
466 and the myc-tagged proteins (SEPT5_v2a or parkin) were
467 immunoprecipitated from the lysates. The parkin and
468 SEPT5_v2a were then combined together along with
469 recombinant UbcH8 and HA-ubiquitin, as well as ATP and
470 buffer. Following 1 h of incubation, the mixture was
471 immunoblotted with either anti-ubiquitin to detect ubiquiti-
472 nated proteins or anti-SEPT5_v2a. SEPT5_v2a that had
473 been incubated with parkin showed the presence of higher
474 molecular weight bands, suggesting that it had been
475 ubiquitinated. The anti-ubiquitin immunoblot showed in-
476 creased levels of high molecular weight ubiquitin conju-
477 gates in lanes containing the SEPT5_v2a (Fig. 4A). Omis-
478 sion of parkin eliminated the high molecular weight bands
479 seen with the SEPT5_v2a or ubiquitin antibodies, and
480 omission of SEPT5_v2a eliminated all reactivity seen with
481 anti-SEPT5_v2a and reduced the high molecular weight
482 ubiquitin conjugated proteins. Omission of SEPT5_v2a
483 would not have been expected to eliminate the high
484 molecular weight ubiquitin proteins because parkin auto-
485 ubiquitinates. In a parallel experiment, we performed in
486 vitro ubiquitination and immunoblotted with antibody to
487 SEPT5_v2a. High molecular weight bands were apparent
488 only in the lanes containing SEPT5_v2a, parkin and other
489 requisite reagents (Fig. 4B). Omission of SEPT5_v2a,
490 parkin, Ubch8, or ubiquitin+E1 eliminated the bands.
491 Quantification of the in vitro ubiquitination reactions is
492 shown in Fig. 4C and D. These data suggest that parkin
493 can ubiquitinate SEPT5_v2a.

494 *3.6. Levels of SEPT5_v1 and SEPT5_v2a are increased*
495 *in brains from patients with ARJP*

496 The ability of parkin to modulate the ubiquitination and
497 turnover of SEPT5_v2a in cell culture suggests that parkin
498 might also regulate turnover of SEPT5_v2a in the brain. To
499 investigate this question, we examined the levels of
500 SEPT5_v2a in brains of patients who died with ARJP.
501 Lysates were obtained from frontal cortex of ARJP brains
502 ($N=4$), and age-matched control brains ($N=5$). Frontal

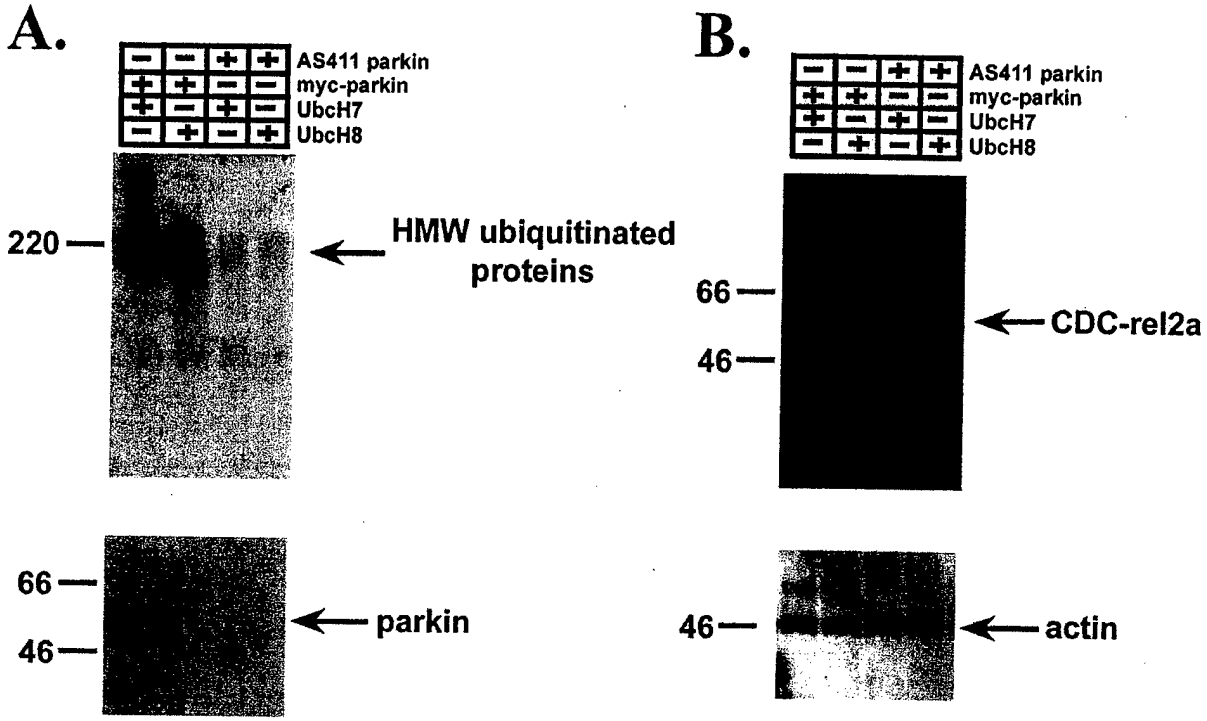


Fig. 3. (A, B) HEK 293 cells were transfected with SEPT5_v2a, parkin (sense or antisense), HA-ubiquitin and UbcH8. Two days after transfection, the cells were harvested and the lysates immunoblotted. (A) The top panel shows immunoblotting with anti-HA (recognizing ubiquitin), and the bottom panel shows immunoblotting with anti-parkin. Ubiquitination paralleled parkin expression. (B) In the same samples, immunoblots were performed with anti-SEPT5_v2a (top panel) and actin (bottom panel). Although the amount of protein did not vary, decreased parkin expression corresponded to increased SEPT5_v2a expression.

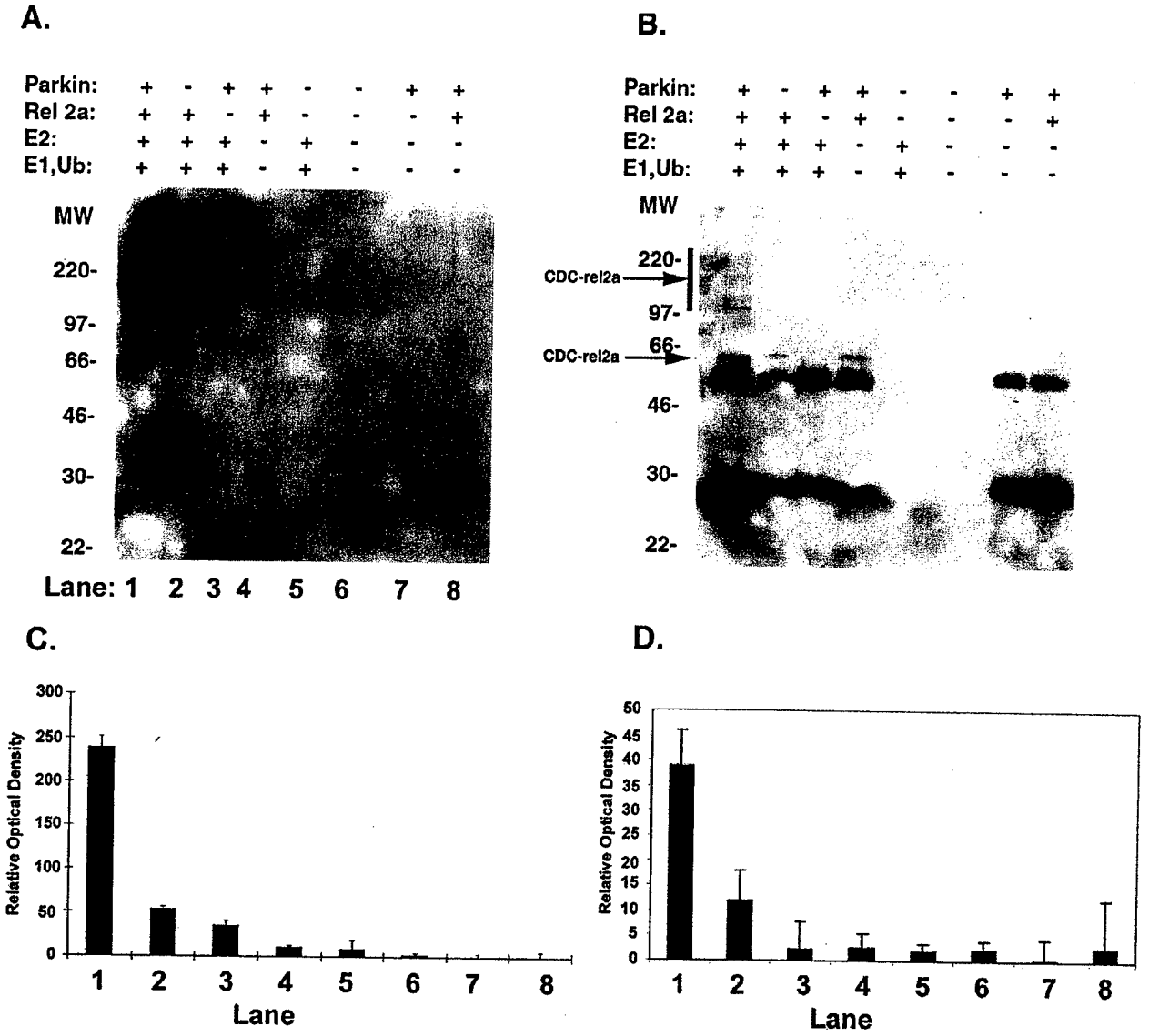
cortex was used because of the limited availability of substantia nigra from ARJP. We felt that analysis of cortical tissue in ARJP might be informative because parkin is expressed throughout the brain. Based on this distribution, loss of parkin might be expected to affect areas other than the substantia nigra, even though pathology (in the form of cell loss) is most severe in the substantia nigra. Consistent with this hypothesis, we observed that the levels of SEPT5_v2a were increased in brains from ARJP donors, although no change was seen in levels of actin (Fig. 5, top panel). Because SEPT5_v1 has also been shown to associate with parkin, we examined whether levels of SEPT5_v1 protein were increased in ARJP brain. We observed that SEPT5_v1 was also increased in three out of four cases of ARJP brain that were examined (Fig. 5, middle panel). No significant differences in actin were observed among the samples (Fig. 5, lower panel). These data suggest that parkin regulates the turnover of both SEPT5_v1 and SEPT5_v2a in vivo.

4. Discussion

Parkin is an E3 ligase, but its substrates are only beginning to be identified. Recently, SEPT5_v1 was shown to bind parkin and have its catabolism regulated by parkin [36]. The protein SEPT5_v2a is a member of the septin family of proteins, and is a close homologue of SEPT5_v1 [9]. We have now shown that parkin also binds SEPT5_v2a, ubiquitinates SEPT5_v2a, and can modulate the levels of SEPT5_v2a in cells. We also observe that levels of both SEPT5_v1 and SEPT5_v2a are increased in the brains of patients with ARJP. Septins are small proteins that can have GTPase activity and appear to function in membrane transport and exocytosis [2,9,26]. The ability of parkin to bind both SEPT5_v1 and SEPT5_v2 suggests a biochemical link between septins and parkin, although the functional consequences of the putative interaction between parkin and SEPT5_v1 proteins remain to be determined.

4.1. Parkin binds to specific domains on SEPT5_v2

Parkin appears to bind SEPT5_v2a at two different regions within the protein, the N-terminal ubiquitin-homology domain and the R1 RING finger domain. The RING finger domains appear to be important for binding and ubiquitination of ligase substrates. For instance, SEPT5, Synphilin-1 and Pael-R bind the R2 RING finger domain, and Tau all bind the R2-IBR domain [6,14,27,36]. In our experiments, we observed different binding patterns depending on whether we examined binding by yeast two-



560

561 Fig. 4. In vitro ubiquitination of SEPT5_v2a by parkin. HEK cells were transfected with either myc-tagged SEPT5_v2a or myc-tagged parkin. The
562 SEPT5_v2a and parkin were immunoprecipitated, combined, an in vitro ubiquitination was performed and the product was immunoblotted for anti-ubiquitin
563 (A) or anti-SEPT5_v2a (B). High molecular weight bands positive for both ubiquitin and SEPT5_v2a were present in samples of SEPT5_v2a that had been
564 co-transfected with parkin, suggesting that parkin lead to ubiquitination of SEPT5_v2a. Omission of any of the reaction components eliminated the
565 ubiquitination. The prominent bands at 25 and 50 kDa represent the heavy and light chains of the anti-myc antibody used to immunoprecipitate the parkin
566 and SEPT5_v2a proteins. Quantification of the in vitro ubiquitination for both ubiquitin reactivity and high molecular weight SEPT5_v2a reactivity is
567 shown in panels (C) and (D), respectively (n=3).

568 hybrid analysis or by immunoprecipitation from mam-
569 malian cells. Binding in the yeast occurs only with the
570 full-length construct or a construct containing only the
571 N-terminal ubiquitin binding domain. Binding in mam-
572 malian cells is detected with constructs coding for either
573 the N-terminal ubiquitin binding domain or constructs
574 containing the RING1-IBR domain. Because other parkin
575 substrates bind to the RING-IBR domains, the immuno-
576 precipitation experiments appear more likely to reflect the
577 true association that occurs in mammals. Several studies
578 show that the RING-IBR-RING domain is required for
579 ubiquitin ligase activity, which suggests that binding of
580 SEPT5_v2 to this region is important for the ability of
581 parkin to ubiquitinate the protein [36].

582 The N-terminal ubiquitin homology domains of ligases
583 are important for the binding of ligases to other proteins in
584 the ubiquitin proteasomal cascade. For instance, the
585 ubiquitin homology domain is important for binding of
586 ligases to proteasomal proteins [33]. The N-terminal
587 ubiquitin homology domain of parkin also appears to be
588 required for parkin function because mutations in this
589 domain block the ubiquitin ligase activity of parkin, and

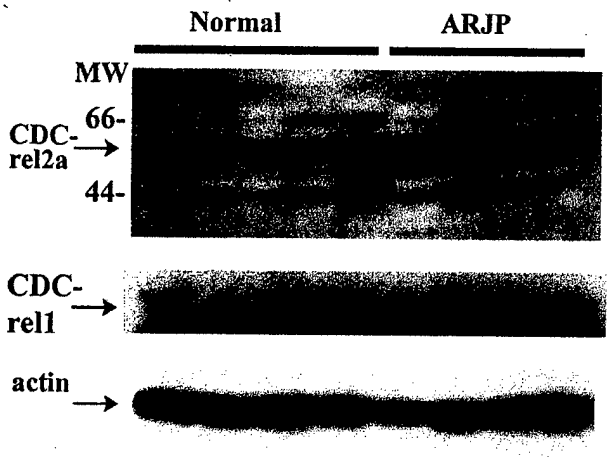


Fig. 5. Levels of SEPT5_v1 and SEPT5_v2 are increased in brains of patients with ARJP. Samples of cortex from cases of ARJP and age-matched controls were immunoblotted with anti-SEPT5_v2a (top panel), SP20 (middle panel), or anti-actin (bottom panel). Although the levels of actin were constant among the samples, the levels of SEPT5_v1 and 2a were increased in the ARJP brains.

are associated with ARJP [1,31,36]. By binding to the ubiquitin homology domain of parkin, SEPT5_v2a could modulate the association of parkin with other proteins or cellular organelles, such as the proteasome.

4.2. Parkin and disease

One of the critical tests of whether a protein is a substrate of parkin is determining whether loss of parkin function increases the steady state level of the protein. The levels of several parkin substrates, including glycosylated α-synuclein, synphilin-1 and Pael-R, are all increased in brains from ARJP donors [6,14,32]. We observed a similar phenomenon for both SEPT5_v1 and SEPT5_v2. Brains from subjects with ARJP show increased levels of SEPT5_v1 and SEPT5_v2 compared to controls, although SEPT5_v1 was increased in only three out of four ARJP brains examined. Unfortunately, insufficient knowledge is available to determine how elevated levels of SEPT5_v1 and SEPT5_v2 might contribute to the pathophysiology of ARJP.

Although mutations in parkin are associated with ARJP, parkin also appears to contribute to the pathophysiology of PD. Some parkin mutations are also associated with familial forms of PD that are indistinguishable from sporadic PD [1]. In addition, parkin accumulates in Lewy bodies [4,5,30]. The mechanism by which parkin accumulates in Lewy bodies is unknown, but could derive from the association of parkin with other proteins that accumulate in Lewy bodies. Shimura and colleagues observed that parkin associates with α-synuclein, but they observed that parkin only binds and ubiquitinates a glycosylated form of α-synuclein that represents only about 1% of the total α-synuclein pool [32]. Parkin also binds to other proteins that are present in LB, such as synphilin and cytochrome *c* [7,11]. Synphilin was recently shown to also bind and be a substrate for parkin [6]. Binding of parkin to α-synuclein and synphilin raises the possibility that parkin might appear in Lewy bodies due to its binding to synuclein and synphilin. In this context, it is interesting that we did not detect SEPT5_v2a in Lewy bodies (data not shown). The reason for the absence of SEPT5_v2a in Lewy bodies could simply result from antibodies that are not sufficiently sensitive to detect the protein in Lewy bodies. A recent study by Ihara and colleagues did identify septins in Lewy bodies [12]. This observation is consistent with a previous study showing the association of septins with inclusions in neurodegenerative diseases, because prior studies have identified the septins Nedd5, diff6 and H5 in neurofibrillary tangles in Alzheimer's disease [17]. These observations add septins to the list of proteins affected by the pathophysiology of PD.

Increasing evidence suggests that parkin might serve a neuroprotective function in the brain. As an ubiquitin ligase, parkin could promote the degradation of proteins that have been damaged by oxidation or other events that might denature the proteins. Studies show that parkin associates with a multi-protein complex that handles such denatured proteins [15]. Other studies show that over-expressing parkin protects against toxicity induced by α-synuclein in cell culture or in Drosophila [28,34]. Parkin also suppresses the toxicity of the Pael receptor, which is toxic when over-expressed and is present in elevated levels in subjects with ARJP [14,34]. The ability of septins to associate with particular vesicles suggests that they could function to target parkin to particular organelles, which could be important for controlling the proteins with which parkin associates [2]. Although we do not know whether SEPT5_v2 modulates parkin-mediated neuroprotection, the importance of parkin in neuroprotection suggests that this is an important line for further investigation.

References

[1] N. Abbas, C. Lücking, S. Ricard, A. Dürr, V. Bonifati, G. De Michele, S. Bouley, J. Vaughan, T. Gasser, R. Marconi, E. Broussolle, C. Brefel-Courbon, B. Harhangi, B. Oostra, E. Fabrizio, G. Böhme, L. Pradier, N. Wood, A. Filla, G. Meco, P. Denefle, Y. Agid, A. Brice, A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe, Hum. Mol. Genet. 8 (1999) 567–574.

[2] C.L. Beites, H. Xie, R. Bowser, W.S. Trimble, The septin CDCrel-1 binds syntaxin and inhibits exocytosis, Nat. Neurosci. 2 (1999) 434–439.

[3] V. Bonifati, P. Rizzu, M.J. van Baren, O. Schaap, G.J. Breedveld, E. Krieger, M.C. Dekker, F. Squitieri, P. Ibanez, M. Joosse, J.W. van Dongen, N. Vanacore, J.C. van Swieten, A. Brice, G. Meco, C.M. van Duijn, B.A. Oostra, P. Heutink, Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism, Science 299 (2003) 256–259.

[4] P. Choi, N. Golts, H. Snyder, L. Petrucelli, M. Chong, J. Hardy, D.

Sparkman, E. Cochran, J. Lee, B. Wolozin, Co-association of parkin and α-synuclein, NeuroReport 12 (2001) 2839–2844.

[5] P. Choi, N. Ostrerova-Golts, D. Sparkman, E. Cochran, J. Lee, B. Wolozin, Parkin is metabolized by the ubiquitin/proteosomal system, NeuroReport 11 (2000) 2635–2638.

[6] K. Chung, Y. Zhang, K. Lim, Y. Tanaka, H. Huang, J. Gao, C. Ross, V. Dawson, T. Dawson, Parkin ubiquitinates the α-synuclein-interacting protein, Synphilin-1: implications for Lewy body formation in Parkinson's disease, Nature Med. 7 (2001) 1144–1150.

[7] S. Engelender, Z. Kaminsky, X. Guo, A. Sharp, R. Amaravi, J. Kleiderlein, R. Margolis, J. Troncoso, A. Lanahan, P. Worley, V. Dawson, T. Dawson, C. Ross, Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions, Nat. Genet. 22 (1999) 110–114.

[8] M. Farrer, P. Chan, R. Chen, L. Tan, S. Lincoln, D. Hernandez, L. Forno, K. Gwinn-Hardy, L. Petrucelli, J. Hussey, A. Singleton, C. Tanner, J. Hardy, J.W. Langston, Lewy bodies and parkinsonism in families with parkin mutations, Ann. Neurol. 50 (2001) 293–300.

[9] C.M. Field, D. Kellogg, Septins: cytoskeletal polymers or signalling GTPases?, Trends Cell Biol. 9 (1999) 387–394.

[10] B.S. Harhangi, M.J. Farrer, S. Lincoln, V. Bonifati, G. Meco, G. De Michele, A. Brice, A. Durr, M. Martinez, T. Gasser, B. Bereznai, J.R. Vaughan, N.W. Wood, J. Hardy, B.A. Oostra, M.M. Breteler, The Ile93Met mutation in the ubiquitin carboxy-terminal-hydrolase-L1 gene is not observed in European cases with familial Parkinson's disease, Neurosci. Lett. 270 (1999) 1–4.

[11] M. Hashimoto, A. Takeda, L. Hsu, T. Takenouchi, E. Masliah, Role of cytochrome *c* as a stimulator of α-synuclein aggregation in Lewy body disease, J. Biol. Chem. 274 (1999) 28849–28852.

[12] M. Ihara, H. Tomimoto, H. Kitayama, Y. Morioka, I. Akiguchi, H. Shibasaki, M. Noda, M. Kinoshita, Association of the cytoskeletal GTP-binding protein Sept4/H5 with cytoplasmic inclusions found in Parkinson's disease and other synucleinopathies, J. Biol. Chem. (2003).

[13] Y. Imai, M. Soda, S. Hatakeyama, T. Akagi, T. Hashikawa, K.I. Nakayama, R. Takahashi, CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity, Mol. Cell 10 (2002) 55–67.

[14] Y. Imai, M. Soda, H. Inoue, N. Hattori, Y. Mizuno, Y. Takahashi, An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of parkin, Cell 105 (2001) 891–902.

[15] Y. Imai, M. Soda, R. Takahashi, Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity, J. Biol. Chem. 275 (2000) 35661–35664.

[16] B. Kartmann, D. Roth, Novel roles for mammalian septins: from vesicle trafficking to oncogenesis, J. Cell Sci. 114 (2001) 839–844.

[17] A. Kinoshita, M. Kinoshita, H. Akiyama, H. Tomimoto, I. Akiguchi, S. Kumar, M. Noda, J. Kimura, Identification of septins in neurofibrillary tangles in Alzheimer's disease, Am. J. Pathol. 153 (1998) 1551–1560.

[18] T. Kitada, S. Asakawa, N. Hattori, H. Matsumine, Y. Yamamura, S. Minoshima, M. Yokochi, Y. Mizuno, N. Shimizu, Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism, Nature 392 (1998) 605–608.

[19] C. Klein, P.P. Pramstaller, B. Kis, C.C. Page, M. Kann, J. Leung, H. Woodward, C.C. Castellan, M. Scherer, P. Vieregge, X.O. Breakefield, P.L. Kramer, L.J. Ozelius, Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype, Ann. Neurol. 48 (2000) 65–71.

[20] R. Kruger, W. Kuhn, T. Muller, D. Woitalla, M. Graeber, S. Kosel, H. Przuntek, J. Epplen, L. Schols, O. Riess, Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease, Nat. Genet. 18 (1998) 106–108.

[21] A. Lang, A. Lozano, Parkinson's disease, New Engl. J. Med. 339 (1998) 1044–1053.

[22] W.D. Le, P. Xu, J. Jankovic, H. Jiang, S.H. Appel, R.G. Smith, D.K. Vassilatis, Mutations in NR4A2 associated with familial Parkinson disease, Nat. Genet. 33 (2003) 85–89.

[23] E. Leroy, R. Boyer, G. Auburger, B. Leube, G. Ulm, E. Mezey, G. Harta, M. Brownstein, S. Jonnalagada, T. Chernova, A. Dehejia, C. Lavedan, T. Gasser, P. Steinbach, K. Wilkinson, M. Polymeropoulos, The ubiquitin pathway in Parkinson's disease, Nature 395 (1998) 451–452.

[24] C.B. Lucking, N. Abbas, A. Durr, V. Bonifati, A. Bonnet, T. de Broucker, G. De Michele, N.W. Wood, Y. Agid, A. Brice, Homozygous deletions in parkin gene in European and North African families with autosomal recessive juvenile parkinsonism, Lancet 352 (1999) 1355–1356.

[25] C.B. Lucking, A. Durr, V. Bonifati, J. Vaughan, G. De Michele, T. Gasser, B.S. Harhangi, P. Pollak, A.M. Bonnet, D. Nichol, M. DeMari, R. Marconi, E. Broussolle, O. Rascol, M. Rosier, I. Arnould, B.A. Oostr, M. Breteler, A. Filla, G. Meco, P. Denefle, N.W. Wood, Y. Agid, A. Brice, Association between early-onset parkinson's disease and mutations in the parkin gene, New Engl. J. Med. 342 (2000) 1560–1567.

[26] I.G. Macara, R. Baldarelli, C.M. Field, M. Glotzer, Y. Hayashi, S.C. Hsu, M.B. Kennedy, M. Kinoshita, M. Longtine, C. Low, L.J. Maltais, L. McKenzie, T.J. Mitchison, T. Nishikawa, M. Noda, E.M. Petty, M. Peifer, J.R. Pringle, P.J. Robinson, D. Roth, S.E. Russell, H. Stuhlmann, M. Tanaka, T. Tanaka, W.S. Trimble, J. Ware, N.J. Zeleznik-Le, B. Zieger, Mammalian septins nomenclature, Mol. Biol. Cell 13 (2002) 4111–4113.

[27] L. Petrucelli, K. Kehoe, Y. Tsuboi, T. Ishizawa, D. Dickson, R. Ved, J. Lewis, E. McGowan, G. Gonzalez-de-Chavez, P. Lockhart, C. O'Farrell, D. Hernandez, M. Van Slegtenhorst, M. Cookson, J. Lee, E. Cochran, P. Choi, M. Farrer, N. Hattori, Y. Mizuno, K. Chung, K. Lim, Y. Zhang, T. Dawson, J. Hardy, M. Hutton, B. Wolozin, Tau is a target for the E3 ligase activity of CHIP and parkin (submitted for publication).

[28] L. Petrucelli, C. O'Farrell, P.J. Lockhart, M. Baptista, K. Kehoe, L. Vink, P. Choi, B. Wolozin, M. Farrer, J. Hardy, M.R. Cookson, Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons, Neuron 36 (2002) 1007–1019.

[29] M.H. Polymeropoulos, C. Lavedan, E. Leroy, S.E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubenstein, R. Boyer, E.S. Stenroos, S. Chandrasekharappa, A. Athanassiadou, T. Papapetropoulos, W.G. Johnson, A.M. Lazzarini, R.C. Duvoisin, G. Di Iorio, L.I. Golbe, R.L. Nussbaum, Mutation in the alpha-synuclein gene identified in families with Parkinson's disease, Science 276 (1997) 2045–2047.

[30] M.G. Schlossmacher, M.P. Frosch, W.P. Gai, M. Medina, N. Sharma, L. Forno, T. Ochiishi, H. Shimura, R. Sharon, N. Hattori, J.W. Langston, Y. Mizuno, B.T. Hyman, D.J. Selkoe, K.S. Kosik, Parkin localizes to the lewy bodies of Parkinson disease and dementia with lewy bodies, Am. J. Pathol. 160 (2002) 1655–1667.

[31] H. Shimura, N. Hattori, S. Kubo, Y. Mizuno, S. Asakawa, S. Minoshima, N. Shimizu, K. Iwai, T. Chiba, K. Tanaka, T. Suzuki, Familial parkinson disease gene product, parkin, is a ubiquitin-protein ligase, Nat. Genet. 25 (2000) 302–305.

[32] H. Shimura, M.G. Schlossmacher, N. Hattori, M.P. Frosch, A. Trockenbacher, R. Schneider, Y. Mizuno, K.S. Kosik, D.J. Selkoe, Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease, Science 293 (2001) 263–269.

[33] K.J. Walters, M.F. Kleijnen, A.M. Goh, G. Wagner, P.M. Howley, Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a, Biochemistry 41 (2002) 1767–1777.

[34] Y. Yang, I. Nishimura, Y. Imai, R. Takahashi, B. Lu, Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila, Neuron 37 (2003) 911–924.

[35] D.M. Zhang, D. Levitan, G. Yu, M. Nishimura, F. Chen, A. Tandon, T. Kawarai, S. Arawaka, A. Supala, Y.Q. Song, E. Rogaeva, Y.

Liang, E. Holmes, P. Milman, C. Sato, L. Zhang, P. St George-Hyslop, Mutation of the conserved N-terminal cysteine (Cys92) of human presenilin 1 causes increased A beta42 secretion in mammalian cells but impaired Notch/lin-12 signalling in *C. elegans*, Neuroreport 11 (2000) 3227–3230.

[36] Y. Zhang, J. Gao, K.K. Chung, H. Huang, V.L. Dawson, T.M. Dawson, Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1, Proc. Natl. Acad. Sci. USA 97 (2000) 13354–13359.

Appendix 3

Mechanisms of degeneration in Parkinson's disease

Benjamin Wolozin*, Mark Frasier, Heather Snyder, Peter Choi, and Natalie Golts

Dept. of Pharmacology, Loyola University Medical Center Maywood, IL

*Corresponding Author

Dept. of Pharmacology

Loyola University Medical Center

Bldg. 102, Room 3634

2160 South 1st Ave.

Maywood, IL 60153

Keywords: α-Synuclein, ubiquitin, protein aggregation, oxidation, fibrillization, microtubule associated protein tau, parkin.

Abbreviations: Parkinson's disease, PD

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease that is the most prevalent movement disorder in the elderly. The symptoms of the disease are tremor, bradykinesia, muscular rigidity and loss of balance. These symptoms primarily result from the degeneration of dopaminergic neurons in the substantia nigra. The neuropathological hallmark of the disease is the Lewy body, which is an intracellular proteinaceous inclusion that accumulates in the remaining neurons of the substantia nigra.

Both genetic and environmental factors appear to contribute to the etiology of PD. Epidemiological studies show that PD is more prevalent among rural farming communities and among factory workers exposed to heavy metals [1-5]. Pesticides, herbicides and fungicides all appear to contribute to PD, and pesticides, such as rotenone, model some aspects of PD in rodent models [3].

Studies of twins with PD suggest a genetic contribution to the etiology of PD [6]. Mutations in several genes have been associated with PD. One protein, termed α-synuclein, exhibits an autosomal dominant mode of inheritance [7, 8]. α-Synuclein is particularly interesting because it appears to be the principle component of Lewy bodies [9, 10]. The autosomal dominant mode of inheritance suggests that the mutation causes a gain of function for α-synuclein. As will be described below, research from a number of laboratories suggests that the particular 'function' that is gained by the mutation in α-synuclein is an increased propensity to aggregate, which leads to Lewy body formation.

The other genes that have been shown to be associated with PD all exhibit a recessive mode of inheritance, which suggests a loss of function. Mutations in parkin, DJ-1 and NURR1 are all associated with recessive Parkinsonisms that resemble some aspects of sporadic PD, but often no the entire pathological spectrum [11-13]. For instance, mutations in parkin are associated with autosomal recessive juvenile Parkinsonism, which is a Parkinsonism that presents in the teenage years, leads to motor deficits and degeneration of the substantia nigra, but lacks Lewy bodies.

Protein Aggregation: A central hypothesis

Protein aggregation is thought to play a critical role in the pathophysiology of many late onset neurodegenerative diseases because each of the diseases is characterized by a protein that accumulates to form an inclusion and in each case the protein that accumulates either has a strong tendency to aggregate or disease-related mutations in the protein tend to increase its tendency to aggregate. β-Amyloid accumulates in Alzheimer's disease, and the disease-related mutations in amyloid precursor protein and presenilins mostly lead to increased production of a β-amyloid that is 42 rather than 40 amino acids long and has a much greater tendency to aggregate. Similarly, mutations in polyglutamine disorders, such as Huntington's disease, lead to production of polyglutamine expansions that aggregate readily, and mutations in frontotemporal dementias of chromosome 17 are caused by mutations in tau protein that increase the tendency of tau to aggregate.

The mutations in α-synuclein that are associated with PD follow a similar pattern. Two mutations are associated with PD, the A53T and the A30P mutations. Each of these mutations increases the tendency of α-synuclein to aggregate. The A53T mutation increases the tendency of α-synuclein to fibrillize, and the A30P mutation increases the tendency of α-synuclein to nucleate [14-18].

Protein aggregation proceeds through a two-step process that starts with nucleation and proceeds to fibrillization (figure 1). The process of protein aggregation can be understood by considering the process of crystallization. Crystallization exhibits a strong lag phase during which nucleation occurs. Nucleation is the rate-limiting step in crystallization. Once nucleation occurs and crystal niduses are generated, crystal formation occurs rapidly. Protein aggregation exhibits similar phenomena. Protein aggregation begins slowly and exhibits a strong lag phase. During the lag phase, small protofibrils form. Once sufficient numbers of protofibrils have formed, the fibrils begin to coalesce to form large fibrils, and the rate of protein aggregation increases greatly (figure 1).

The biphasic process of protein aggregation leads has important implications. Factors that affect the rate of either step will increase the rate of protein aggregation. One factor that has already been mentioned is mutation. Another factor is concentration. Studies in vitro, in cell culture and in vivo show that increasing the concentration of α-synuclein increases the rate of its aggregation [14-22]. Exogenous agents that increase the tendency of α-synuclein to bind to itself also increases the tendency to aggregate. Dopamine, for

instance, increases the tendency of α-synuclein to form protofibrils, and increases the rate of α-synuclein aggregation. Iron also increases the tendency of α-synuclein to fibrillate and also increases the tendency of α-synuclein to aggregate. Finally, free radicals tend to make α-synuclein dimerize, and also increase the tendency of α-synuclein to fibrillate. Hence, agents such as arachadonic acid, hydrogen peroxide and other oxidants increase the rate of α-synuclein fibrillization [16, 23, 24].

Taken together, these factors explain much of the aggregation behavior of α-synuclein. For instance, the tendency of α-synuclein to aggregate in dopaminergic neurons of the substantia nigra might result in part from the abundance of dopamine in the substantia nigra and the tendency of the substantia nigra to accumulate iron with age. Similarly, diseases in which iron accumulates, such as Iron Accumulation Disease, Type I (Hallovorden Spatz disease), show extensive α-synuclein aggregation [25]. The biphasic aggregation process also needs to be considered when designing assays to monitor α-synuclein aggregation. Some monitoring methods, such as the thioflavin T or turbidity assays, primarily detect formation of large fibrils of α-synuclein. Other assays such as ELISA capture assays primarily detect formation of protofibrils. Each assay yields particular kinetic results that depend on the accumulation of the particular aggregation intermediate being studied [26].

Inhibitors of Aggregation

The ability to recapitulate the aggregation process in vivo, enables detection of agents that can inhibit the aggregation process. For instance, while iron promotes α-synuclein

aggregation, magnesium inhibits the process. Magnesium dramatically inhibits both spontaneous and iron-induced α-synuclein aggregation in vitro [27]. Magnesium also inhibits iron-induced α-synuclein aggregation in neurons grown in cell culture (figure 2). To demonstrate this, we took primary cultures of cortical neurons and exposed them to 0.1 mM $FeCl_2$ in the presence of 0-3 mM $MgCl_2$ during 24 hr incubation. We then counted the number of neurons with processes longer than 100 μm. As shown in figure 2, magnesium almost completely inhibited the aggregation of α-synuclein. These results suggest that magnesium might be able to be used to inhibit α-synuclein aggregation in vivo, and that endogenous levels of magnesium in vivo might modify the tendency of α-synuclein to aggregation.

Animal Models: Protein Aggregation in vivo

By combining over-expression with aggregation increasing mutations, investigators have been able to model some aspects of the process of α-synuclein aggregation in vivo. Our studies of α-synuclein aggregation in vivo have utilized a transgenic mouse over-expressing A30P α-synuclein driven by a Thy1 promoter that was developed by Philipp Kahle and Christian Haass [22]. Prior studies show that this mouse model develops a progressive loss of motor function beginning with tremor, which progresses to bradykinesia, then postural instability and ultimately death. The motor decline is accompanied by the accumulation of inclusions in the brain stem that contain aggregated α-synuclein and, to a lesser extent, ubiquitin (figure 3).

The accumulation of inclusions that contain aggregated α-synuclein and ubiquitin in the transgenic A30P α-synuclein mouse partially recapitulates the pathology of PD. However the anatomical distribution of the inclusions does not reflect that of PD because the inclusions accumulate in the brain stem rather than the substantia nigra. The similarities and differences between the pathology of the transgenic mouse and PD subjects suggests that the A30P α-synuclein transgenic mouse might provide a useful model of α-synuclein aggregation but does not provide an model relevant to understanding mechanisms of degeneration specific to dopaminergic neurons.

Using the A30P α-synuclein transgenic mouse, we have begun to explore the pathophysiology of α-synuclein aggregation in vivo. One of the key questions that we have examined first is whether aggregation of α-synuclein stimulates other pathological changes. Increasing data suggests that fibrillization of α-synuclein frequently occurs along with fibrillization of tau protein [28, 29]. This observation prompted us to examine whether tau was affected by the presence of aggregated α-synuclein. To investigate this question, we performed immunocytochemistry with the antibodies PHF1 and AT8, both of which recognize phosphorylated epitopes characteristically observed in neurofibrillary tangles and pre-tangles. We observed that neurons in the ventral brain stem of symptomatic A30P α-synuclein mice stained with both PHF1 and AT8 (figure 4). Approximately 40% of these neurons also contained small foci of α-synuclein staining. Interestingly, the neurons that exhibited the greatest amount of α-synuclein staining occurred in dorsal nuclei of the brain stem. Very little PHF1 or AT8 reactivity was seen in the brain stems from asymptomatic transgenic A30P α-synuclein mice or non-

transgenic mice. We also stained the brains with thioflavin to determine whether the abnormally phosphorylated tau was present as a tangle or a pre-tangle. The neurons that were positive for PHF1 and AT8 did not stain with thioflavin.

The selective appearance of abnormal phosphorylated epitopes in tau protein in symptomatic transgenic A30P α-synuclein mice suggests that aggregation of α-synuclein stimulates pathological changes in tau protein. This could explain why tau pathology occurs alongside α-synuclein pathology in human diseases. The absence of thioflavin staining in the PHF1 and AT8 positive neurons indicates that the tau was present as pretangles rather than as tangles. The pretangle nature of the phosphorylated tau is to be expected because mouse tau protein does not aggregated nearly as quickly as human tau protein. Thus, phosphorylated mouse tau is unlikely to progress to the tangles. The identification of these correlated changes allows us to begin to determine what types of stress kinases are activated by aggregated α-synuclein.

Parkin accumulates in Lewy bodies in PD brains but not in the A30P α-synuclein mouse.

Prior work from suggests that parkin is present in Lewy bodies in brains of subjects with PD. We were curious to determine whether parkin might also occur in the inclusions that accumulate in the A30P α-synuclein mice. Brains from symptomatic A30P α-synuclein mice were stained with either antibody to α-synuclein or to parkin. None of the neurons that accumulated α-synuclein stained positive for parkin. This suggests that α-synuclein aggregation can accumulate without a concomitant accumulation of parkin.

Conclusion

The application of molecular genetics to the study of PD has lead to the identification of a number of genes that participate in the pathophysiology of PD. α-Synuclein has garnered particular interest because it is the only genetic mutation associated with a dominant form of the illness that has been identified to date, and because α-synuclein accumulates in Lewy bodies [7, 9, 10]. Studies in vitro and in vivo show that α-synuclein tends to aggregate, and that mutations in α-synuclein that are associated with PD increase the tendency of α-synuclein to aggregate [15, 16, 22]. Using these α-synuclein models we have shown that factors such as iron and dopamine increase the tendency of α-synuclein to aggregate, while magnesium inhibits α-synuclein aggregation [16]. The studies of α-synuclein aggregation in vivo using the A30P α-synuclein mice show that α-synuclein accumulates in neurons of the brain stem and that this accumulation also elicits the accumulation of ubiquitin reactivity, but not parkin reactivity. Interestingly, aggregation of α-synuclein also stimulates abnormal phosphorylation of tau protein, which is apparent using the PHF1 and AT8 antibodies. Since the phosphorylation events associated with PHF1 and AT8 reactivity are one of the preliminary steps associated with tangle formation, these results suggest that aggregation of α-synuclein moves tau protein towards tangle pathology. These results emphasize the interrelated nature of the different pathologies that occur in neurodegenerative diseases in human illnesses.

There remains an important mechanistic gap between studies of environmental factors implicated in PD, and genetic factors implicated in PD. Studies with rotenone suggest

that prolonged application stimulates selective degeneration of the substantia nigra and formation of α-synuclein positive inclusions [30, 31]. In contrast, over-expressing α-synuclein in a transgenic mouse stimulates α-synuclein aggregation but not in the substantia nigra [19-22]. The identification of proteins such as α-synuclein that are specifically implicated in the neurodegeneration associated with PD provides an important target for pharmaceutical intervention. However, the disparity between the pathology elicited by α-synuclein over-expression and the pathology of PD presents an important challenge for applying this new knowledge to identification of novel therapeutic approaches for the treatment of PD.

Acknowledgements: This work was supported by grants from NIH (NIA, NINDS) and USAMRC.

Figure 1: The biphasic aggregation of α-synuclein begins with a lag phase during which time nucleation occurs generating protofibrils. This process is quite slow. Following formation of sufficient protofibrils, fibrillization occurs rapidly.

Figure 2: Magnesium inhibits the aggregation of α-synuclein in primary cortical neurons. Primary cortical neuronal cultures were exposed to 1 mM $FeCl_2 \pm 0 - 1.5$ mM MgCl for 72 hrs.and then the numberof processes over 10 μm in length= was quantified.

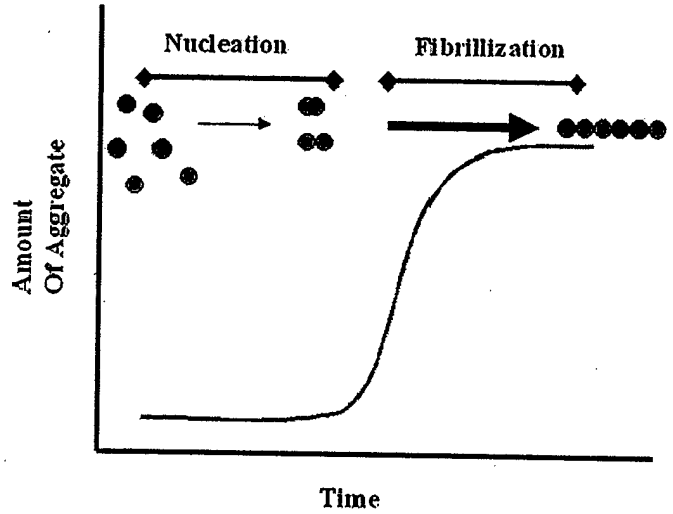
Figure 3: The accumulation of α-synuclein and ubiquitin inclusions in the brains of A30P α-synuclein mice partially recapitulates the pathology of PD. A. Staining of α-synuclein in the brain stem of a symptomatic transgenic A30P mouse. B. Staining of α-synuclein in the brain stem of a non-transgenic age-matched mouse.

Figure 4: Phosphorylated tau epitopes appear in the brain stem of symptomatic A30P α-synuclein transgenic mice. Neurons that stain positive with antibodies to PHF1 appear in symptomatic A30P α-synuclein transgenic mice and co-localize with α-synuclein reactivity. A. α-Synuclein staining. B. PHF1 staining. C. Merge.

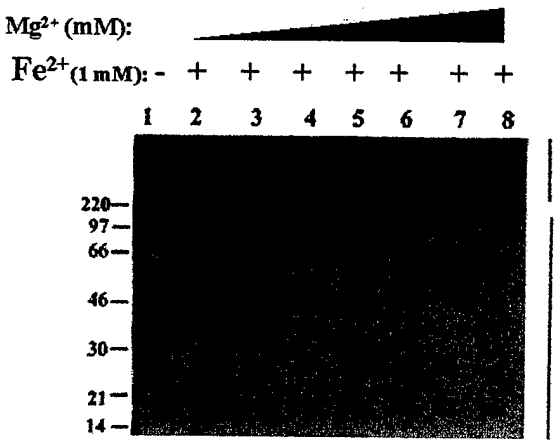
References:

1. Langston, J., Epidemiology versus genetics in Parkinson's disease: Progress in resolving an age-old debate. Ann. Neurol., 1998; 44: p. S45-52.
2. Semchuk, K.M., E.J. Love, and R.G. Lee, Parkinson's disease and exposure to rural environmental factors: a population based case-control study. Can J Neurol Sci, 1991; 18: p. 279-86.
3. Liou, H.H., et al., Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. Neurology, 1997; 48: p. 1583-8.
4. Gorell, J., et al., Occupational metal exposures and the risk of Parkinson's disease. Neuroepidemiology, 1999; 18: p. 303-8.
5. Rybicki, B.A., et al., Parkinson's disease mortality and the industrial use of heavy metals in Michigan. Mov Disord, 1993; 8: p. 87-92.
6. Tanner, C., et al., Parkinson disease in twins: an etiologic study. JAMA, 1999; 281: p. 341-6.
7. Polymeropoulos, M.H., et al., Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science, 1997; 276: p. 2045-7.
8. Kruger, R., et al., Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. Nature Gen., 1998; 18: p. 106-8.
9. Spillantini, M., et al., α-Synuclein in Lewy bodies. Nature, 1997; 388: p. 839-40.
10. Spillantini, M.G., et al., α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc. Natl. Acad. Sci. USA, 1998; 95: p. 6469-73.
11. Kitada, T., et al., Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature, 1998; 392: p. 605-8.
12. Bonifati, V., et al., Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science, 2003; 299: p. 256-9.
13. Le, W.D., et al., Mutations in NR4A2 associated with familial Parkinson disease. Nat Genet, 2003; 33: p. 85-9.
14. Conway, K., J. Harper, and P. Lansbury, Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nature Med., 1998; 4: p. 1318-20.
15. Conway, K.A., et al., Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: Implications for pathogenesis and therapy. Proc Natl Acad Sci U S A, 2000; 97: p. 571-576.
16. Ostrerova-Golts, N., et al., The A53T α-Synuclein Mutation Increases Iron-Dependent Aggregation and Toxicity. J. Neuroscience, 2000; 20: p. 6048-54.
17. Hashimoto, M., et al., Human recombinant NACP/α-synuclein is aggregated and fibrillated in vitro: Relevance for Lewy body disease. Brain Res., 1998; 799: p. 301-6.

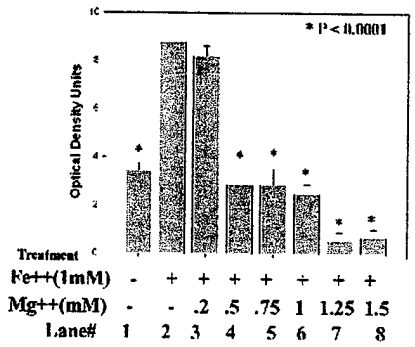
18. Hashimoto, M., et al., Oxidative stress induces amyloid-like aggregate formation of NACP/α-synuclein *in vitro*. NeuroReport, 1999; 10: p. 717-21.
19. Masliah, E., et al., Dopaminergic Loss and Inclusion Body Formation in alpha-Synuclein Mice: Implications for Neurodegenerative Disorders. Science, 2000; 287: p. 1265-1269.
20. Giasson, B.I., et al., Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron, 2002; 34: p. 521-33.
21. Lee, M., et al., Human α-synuclein-harboring familial Parkinson's disease-linked Ala-53 to Thr mutation causes neurodegenerative disease with α-synuclein aggregation in transgenic mice. PNAS, 2002; 99.
22. Kahle, P.J., et al., Selective insolubility of alpha-synuclein in human Lewy body diseases is recapitulated in a transgenic mouse model. Am J Pathol, 2001; 159: p. 2215-25.
23. Perrin, R.J., et al., Exposure to long chain polyunsaturated Fatty acids triggers rapid multimerization of synucleins. J Biol Chem, 2001; 276: p. 41958-62.
24. Perrin, R.J., et al., Interaction of human α-synuclein and Parkinson's disease variants with phospholipids:structural analysis using site-directed mutagenesis. J Biol Chem, 2000; 275: p. 34393-8.
25. Galvin, J.E., et al., Neurodegeneration with brain iron accumulation, type 1 is characterized by alpha-, beta-, and gamma-synuclein neuropathology. Am J Pathol, 2000; 157: p. 361-8.
26. El-Agnaf, O., et al., Effects of the mutations Ala30 to Pro and Ala53 to Thr on the physical and morphological properties of α-synuclein protein implicated in Parkinson's disease. FEBS Lett., 1998; 440: p. 67-70.
27. Golts, N., et al., Magnesium inhibits spontaneous and iron-induced aggregation of alpha- synuclein. J Biol Chem, 2002; 277: p. 16116-23.
28. Ishizawa, T., et al., Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. J Neuropathol Exp Neurol, 2003; 62: p. 389-97.
29. Giasson, B.I., et al., Initiation and synergistic fibrillization of tau and alpha-synuclein. Science, 2003; 300: p. 636-40.
30. Betarbet, R., et al., Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci, 2000; 3: p. 1301-1306.
31. Sherer, T.B., et al., An in vitro model of Parkinson's disease: linking mitochondrial impairment to altered alpha-synuclein metabolism and oxidative damage. J Neurosci, 2002; 22: p. 7006-15.

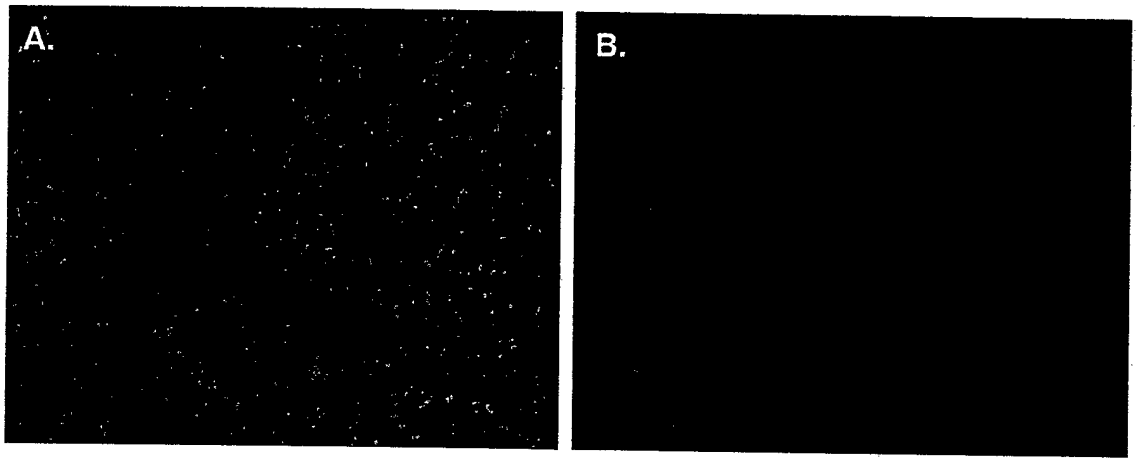

Nucleation
Fibrillization
Amount
Of Aggregate
Time

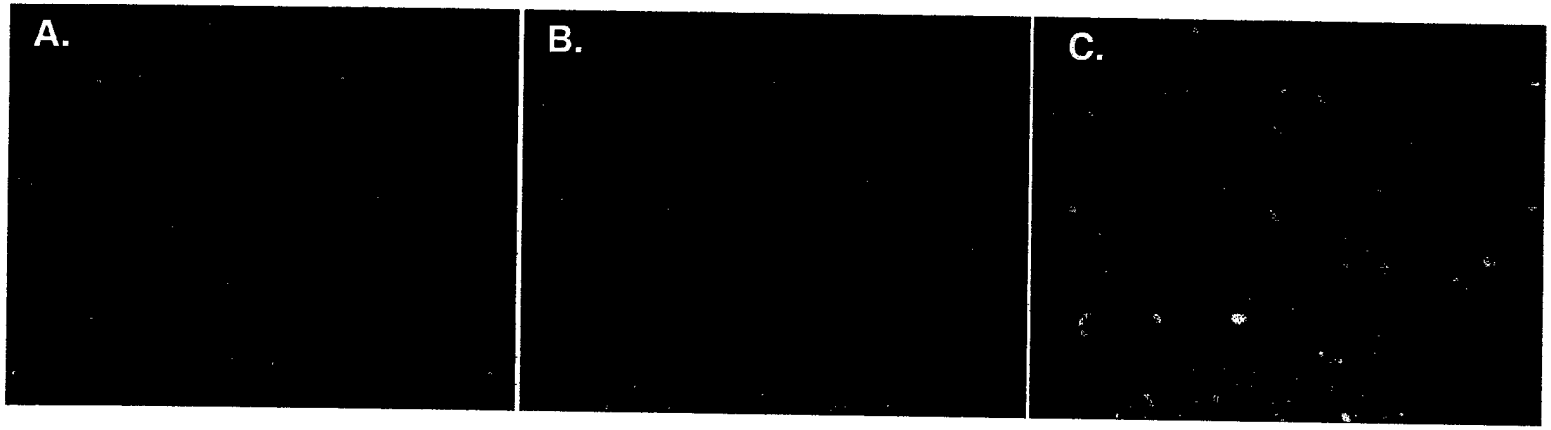
A.


Mg2+ (mM):
Fe2+(1 mM): - + + + + + + +
1 2 3 4 5 6 7 8
220
97
66
46
30
21
14

B.


* P < 0.0001
Optical Density Units
Treatment
Fe++(1mM) - + + + + + + +
Mg++(mM) - - .2 .5 .75 1 1.25 1.5
Lane# 1 2 3 4 5 6 7 8


A.
B.


A.
B.
C.

Appendix 4

Neuron, Vol. 36, 1007–1019, December 19, 2002, Copyright ©2002 by Cell Press

Parkin Protects against the Toxicity Associated with Mutant α-Synuclein: Proteasome Dysfunction Selectively Affects Catecholaminergic Neurons

Leonard Petrucelli,[1] Casey O'Farrell,[1] Paul J. Lockhart,[1] Melisa Baptista,[3] Kathryn Kehoe,[1] Liselot Vink,[1] Peter Choi,[2] Benjamin Wolozin,[2] Matthew Farrer,[1] John Hardy,[3] and Mark R. Cookson[3,4]
[1]Neurogenetics Laboratory
Mayo Clinic Jacksonville
Jacksonville, Florida 32224
[2]Department of Pharmacology
Loyola University Medical Center
Maywood, Illinois 60153
[3]Laboratory of Neurogenetics
National Institute on Aging
Bethesda, Maryland 20892

Summary

One hypothesis for the etiology of Parkinson's disease (PD) is that subsets of neurons are vulnerable to a failure in proteasome-mediated protein turnover. Here we show that overexpression of mutant α-synuclein increases sensitivity to proteasome inhibitors by decreasing proteasome function. Overexpression of parkin decreases sensitivity to proteasome inhibitors in a manner dependent on parkin's ubiquitin-protein E3 ligase activity, and antisense knockdown of parkin increases sensitivity to proteasome inhibitors. Mutant α-synuclein also causes selective toxicity to catecholaminergic neurons in primary midbrain cultures, an effect that can be mimicked by the application of proteasome inhibitors. Parkin is capable of rescuing the toxic effects of mutant α-synuclein or proteasome inhibition in these cells. Therefore, parkin and α-synuclein are linked by common effects on a pathway associated with selective cell death in catecholaminergic neurons.

Introduction

The identification of genes linked to familial forms of Parkinson's disease (PD) provides an important tool for modeling the pathways leading to neurodegeneration in this disorder. To date, eight linkages have been reported, with three genes identified as causal, or probably causal, in different families. Two of these encode proteins whose function is related to ubiquitin-dependent protein degradation through the proteasome (for review, see Hershko and Ciechanover, 1998). Parkin (OMIM 600116) is an E2-dependent E3 protein-ubiquitin ligase (Shimura et al., 2000; Zhang et al., 2000), and mutations in this gene are generally associated with recessive early onset parkinsonism (Kitada et al., 1998). *Parkin* mutations reported to date appear to be loss-of-function mutations reducing the ability of parkin to regulate degradation of substrate removal (Shimura et al., 2000; Zhang et al., 2000). One mutation in ubiquitin-C-terminal hydrolase (*UCHL1*, OMIM 191342) has been described (Farrer et al., 2000; Leroy et al., 1998), although pathogenicity of this mutation has not been fully established. However, polymorphic variability in *UCHL1* has been associated with altered risk for development of PD in case-control studies (Maraganore et al., 1999; Wintermeyer et al., 2000).

Mutations in α-*synuclein* have been reported in autosomal dominant pedigrees (OMIM 601508, Kruger et al., 1998; Polymeropoulos et al., 1997). Several pieces of evidence suggest that α-*synuclein* mutations and proteasome function may be related. Whether α-synuclein turnover is regulated by proteasome function is controversial, with both positive (Bennett et al., 1999; Tofaris et al., 2001) and negative (Ancolio et al., 2000; Paxinou et al., 2001) results reported. Forced overexpression of α-synuclein, especially mutant forms, sensitize PC12 (Stefanis et al., 2001; Tanaka et al., 2001), NT2, and SK-N-MC (Lee et al., 2001b) neuroblastoma cell lines to toxicity induced by the proteasome inhibitor lactacystin. The mechanism by which this occurs is not clear, but overexpression of mutant α-synuclein produces an inhibition of proteasome-associated proteolytic activities. The A30P mutant α-synuclein inhibits the postacidic proteasome activity by 25% and the trypsin-like and chymotrypsin-like activities by slightly smaller amounts, with wild-type α-synuclein having a much smaller effect (Tanaka et al., 2001). The A53T mutant form of α-synuclein also inhibits the chymotrypsin-like activity of the proteasome (Stefanis et al., 2001). Finally, it has been reported that an O-glycosylated form of α-synuclein found in human brain is ubiquitinated by parkin (Shimura et al., 2001), raising the possibility that loss of parkin function might result in α-synuclein accumulation. α-synuclein-positive Lewy bodies have been noted in a case of Parkin-related PD (Farrer et al., 2001).

Overall, the above studies suggest that proteasome inhibition might be a common link between the different genetic triggers of PD. Furthermore, there is evidence that proteasome function is impaired in sporadic PD (McNaught and Jenner, 2001). However, the hypothesis that proteasome dysfunction is an explanation for PD remains conjecture. For example, as cell loss in PD is not uniform, any attempt to link proteasome function to disease should account for selective vulnerability of specific subgroups of neurons. The selective vulnerability of different neuronal types to cell death or formation of the pathological hallmarks of the disease is complex (reviewed in Braak and Braak, 2000), but it is clear that functional loss of dopaminergic neurons in the substantia nigra (SN) *pars compacta* is important. The movement-related symptoms of PD patients are related to dopaminergic cell loss, and loss of these cells not only precedes symptom development, it is also progressive throughout the course of the disease (Pakkenberg et al., 1991).

In the present study we have explored the relationship between overexpression of α-synuclein and parkin with toxicity associated with proteasome inhibition. We have also used primary cell cultures to distinguish effects on

[4]Correspondence: cookson@mail.nih.gov

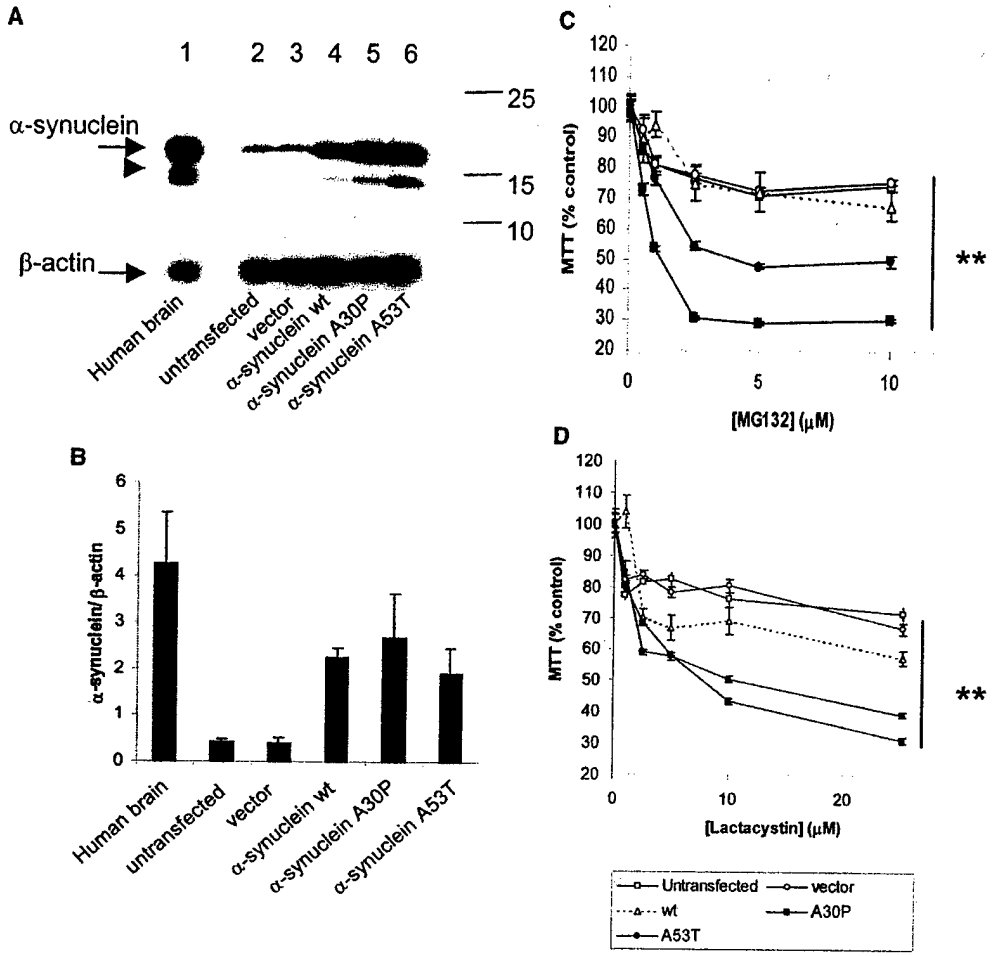


Figure 1. Effects of Proteasome Inhibitors on Cell Viability in M17 Cells Overexpressing α-Synuclein

(A and B) Stable clonal cell lines overexpressing α-synuclein were established and expression screened using Western blotting using monoclonal antibody 42. Untransfected cells (lane 2) or cells transfected with vector alone (lane 3) show moderate expression of α-synuclein compared to cells overexpressing wild-type (lane 4), A30P (lane 5), or A53T synuclein (lane 6). Human cerebral cortex extract was used as a positive control (lane 1). A reprobe of the same blot using β-actin is shown below to demonstrate similarity of loading across the lanes. Quantitation of α-synuclein expression is shown in (B) and is expressed as a ratio of the major synuclein band to β-actin (n = 4 serial passages of the cells, error bars represent the SEM).

(C and D) Overexpression of α-synuclein produces increased sensitivity to the proteasome inhibitor MG132 (C) or lactacystin (D). Cells were exposed to either inhibitor for 24 hr, after which cell viability was estimated using the MTT assay (see Experimental Procedures). Cell lines included untransfected cells (open squares) or cells transfected with empty vector (open circles), WT (open triangles), A30P (closed squares), or A53T (closed circles) α-synuclein. Results are expressed as a percentage of untreated cells for each clonal line (n = 8, each curve is representative of three or more experiments). Similar increased sensitivity to proteasome inhibition was seen in a second set of clonal lines. Statistical significance was estimated using two-way ANOVA using cell lines and concentration of inhibitors as independent variables. **$p < 0.001$ for differences between cell lines, both inhibitors having a significant effect on viability at $p < 0.001$. Representative data from one of three experiments.

tyrosine hydroxylase (TH)-positive neurons compared to TH-negative neurons. We show that parkin and mutant α-synuclein have opposite effects on neuronal cell death associated with impaired proteasome function and that parkin is capable of reducing toxicity associated with α-synuclein overexpression. We also show that knockdown of parkin increases the sensitivity of cells to proteasome inhibition, suggesting that loss-of-function mutations in parkin would cause cell death by the same mechanism as gain-of-function α-synuclein mutations. Furthermore, the effects of either mutant α-synuclein or proteasome inhibition are both selective for TH-positive neurons.

Results

Overexpression of α-Synuclein and Sensitivity to Proteasome Inhibitors

We used human M17 neuroblastoma cells to explore the relationship between mutant forms of α-synuclein and proteasome function. Cell lines stably overexpressing wild-type or either of the two mutants expressed about a 5-fold increased level of α-synuclein compared with untransfected cells (Figure 1A). Although the expression levels are high compared with untransfected cells, α-synuclein protein expression in human brain was 9-fold higher than untransfected M17 cells and approxi-

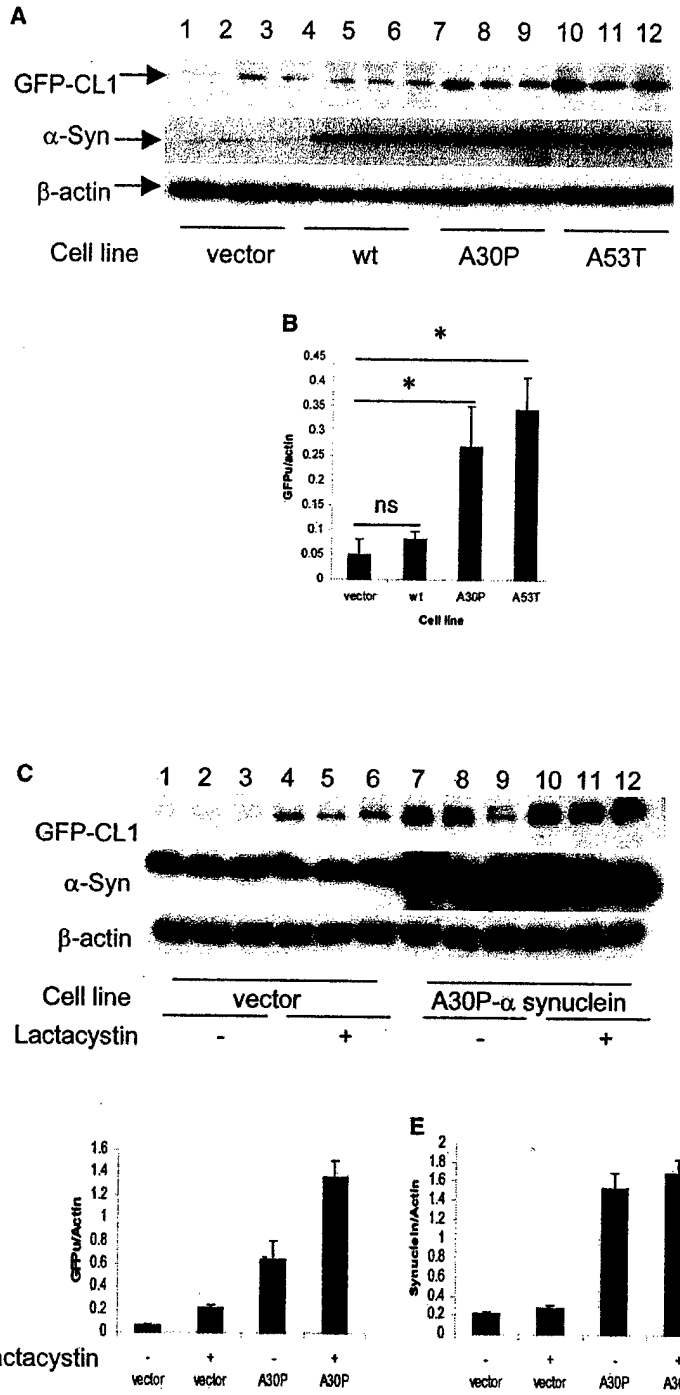


Figure 2. Measurements of Proteasome Activity in Living Cells

(A and B) Overexpression of mutant α-synuclein decreases proteasome activity. Stable cell lines (as in Figure 1) were transiently transfected with the GFP^u construct (see text for description of the construct). These included vector (lanes 1–3), cells expressing wild-type α-synuclein (4–6), A30P (7–9), or A53T (10–12) mutant α-synuclein. Protein extracts were blotted for GFP-CL1 peptide using anti-GFP (top panel) then reprobed sequentially for α-synuclein (middle panel) and β-actin (bottom panel). A semiquantitative analysis was performed by densitometry, correcting GFP-CL1 levels for β-actin. Each bar is the mean of the samples shown in (A) plus a duplicate set (hence n = 6), and error bars indicate the SEM. Both mutant forms of synuclein increased the amount of reporter construct, which was significant between cell lines at $p < 0.001$ by ANOVA.

(C–E) Additive effects of mutant α-synuclein expression and proteasome inhibition. (C) Vector (lanes 1–6) or A30P mutant α-synuclein cell lines (7–12) were transfected with GFP^u then, after 48 hr, treated with 5 μM lactacystin for 6 hr. Protein extracts were blotted for GFP, α-synuclein, and β-actin (top, middle, and bottom panels, respectively) and semiquantitative analysis for GFP immunoreactivity (D) or α-synuclein (E) normalized to β-actin for the same gel, bars are the mean of the samples shown in (C) plus a duplicate set (hence n = 6), and error bars indicate the SEM. Similar results were obtained in duplicate experiments.

mately 30% higher than the highest expressing clone of A30P cells. We also generated a second set of lines with similar levels of expression (data not shown).

We examined the sensitivity of these cell lines to toxicity induced by proteasome inhibition (Figure 1C). Exposure to 10 μM MG132 caused cell viability to be decreased to 74% ± 1% of untreated for untransfected cells and 76% ± 0.8% for vector cells (n = 8). For wild-type α-synuclein transfectants, a small increase in sensitivity was noted at the highest tested concentration, where cell viability was decreased to 68% ± 5%. Cells transfected with either of the mutant forms of α-synuclein were much more sensitive to MG132. The largest effect was seen in cells overexpressing A30P where cell viability was 30% ± 0.4% at 10 μM MG132. Overexpression of A53T α-synuclein decreased cell viability to 50% ± 1% at the same concentration. Similar results were seen with a second set of clones. For example, using two independent A30P-expressing clonal lines, cell viability after exposure to 10 μM MG132 was de-

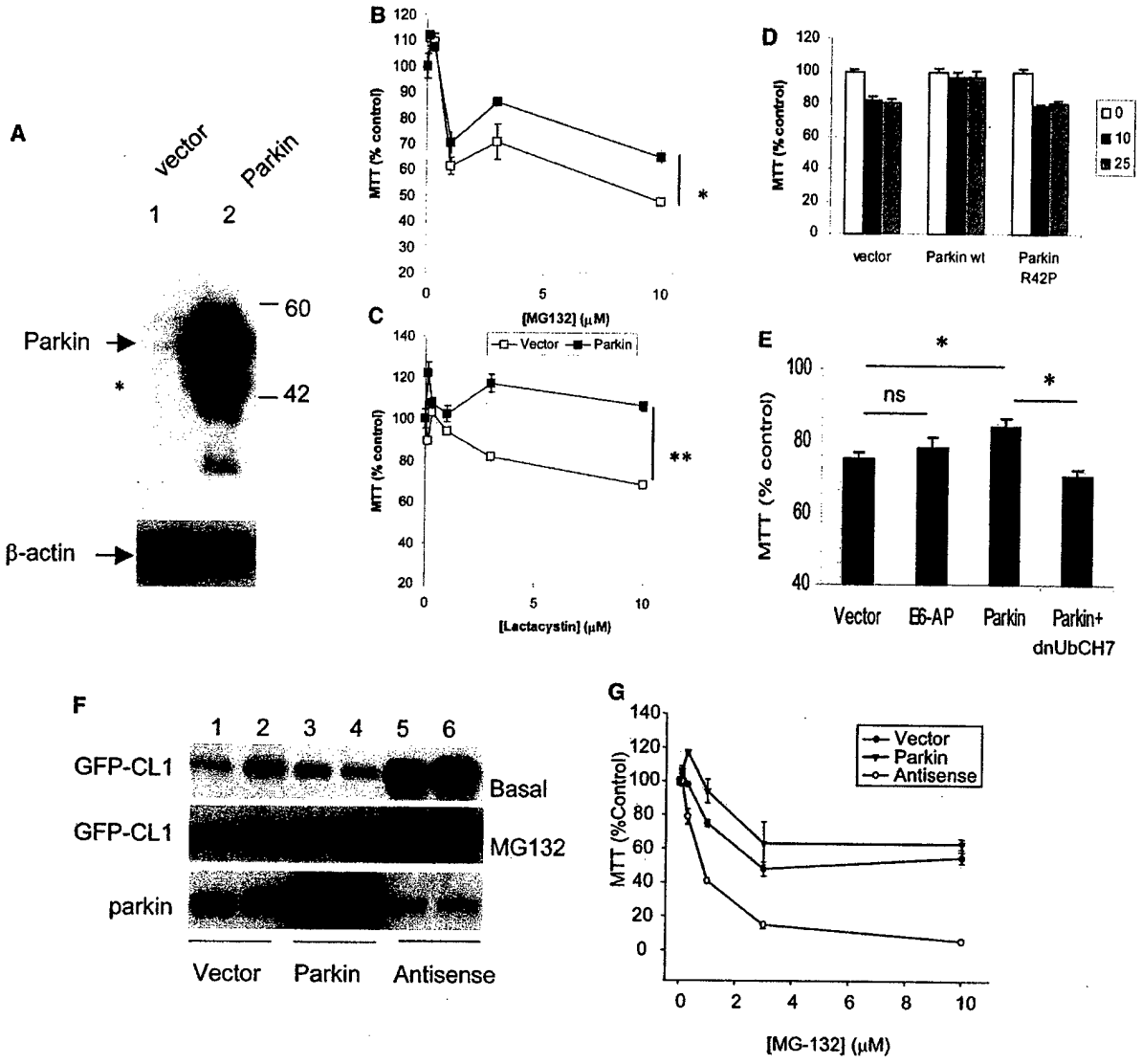


Figure 3. Effects of Proteasome Inhibitors on Cell Viability in M17 Cells Overexpressing Parkin

(A) Parkin protein was measured by Western blotting using a C-terminal antibody to human parkin in clonal lines transfected with vector (lane 1) or expression vector for human parkin (lane 2). Full-length parkin runs at ~50 kDa (arrow) although a C-terminal fragment (~42 kDa) is also noted in the overexpressing cell line (asterisk). Equal loading was demonstrated by reprobing the same blot with β-actin.

(B and C) Overexpression of parkin protects cells against the toxic effects of the proteasome inhibitor MG132 (B) or lactacystin (C). Cells stably transfected with parkin (open symbols) or vector (closed symbols) were exposed to inhibitors for 24 hr and cell viability assessed as in Figure 4. Statistical significance was estimated using two-way ANOVA using cell lines and concentration of inhibitors as independent variables. *$p < 0.05$; **$p < 0.001$ for differences between cell lines, both inhibitors having a significant effect on viability at $p < 0.001$. Similar protective effects of parkin overexpression were seen in two independent experiments (each $n = 8$).

(D) In an independent set of experiments, cells overexpressing mutant Parkin (R42P) were not protected from exposure to 10 μM (filled bars) or 25 μM (striped bars) lactacystin. ($n = 8$, representative of two experiments.)

(E) Cells were transfected with vector, the E3 ligase E6-AP, Parkin alone, or Parkin in the presence of a dominant-negative inhibitor of the E2 enzyme UbCH7 and exposed to 10 μM lactacystin. Cell death was quantified as above ($n = 8$) and expressed as a percentage of MTT conversion for transfected cells that had not been exposed to lactacystin. The differences between vector and parkin transfected cells were significant.

(F) Antisense knockdown of Parkin increases steady-state levels of heterologous substrates. M17 cells stably transfected with wild-type Parkin (lanes 3 and 4) or an antisense parkin construct (lanes 5 and 6) were transiently transfected with GFP^u reporter as above (upper panel) or transfected and then treated with MG132 (middle panel). Vector-only cells were included as controls (lanes 1 and 2, duplicate clonal lines). Parallel samples were blotted for parkin (lower panel) to demonstrate the level of overexpression and the effect of antisense knockdown. GFP-CL1 levels were unaffected by expression of parkin (similar effects were seen in two independent experiments), although antisense parkin cell lines did show an accumulation of GFP-CL1.

(G) MTT assays in the same cell lines show that cells transfected with antisense parkin (open circles) are more sensitive to MG132 toxicity compared to vector-only lines (closed circles) or cells transfected with WT parkin (closed triangles). Results are expressed as a percentage of untreated cells for each clonal line ($n = 8$, each curve is representative of two or more experiments).

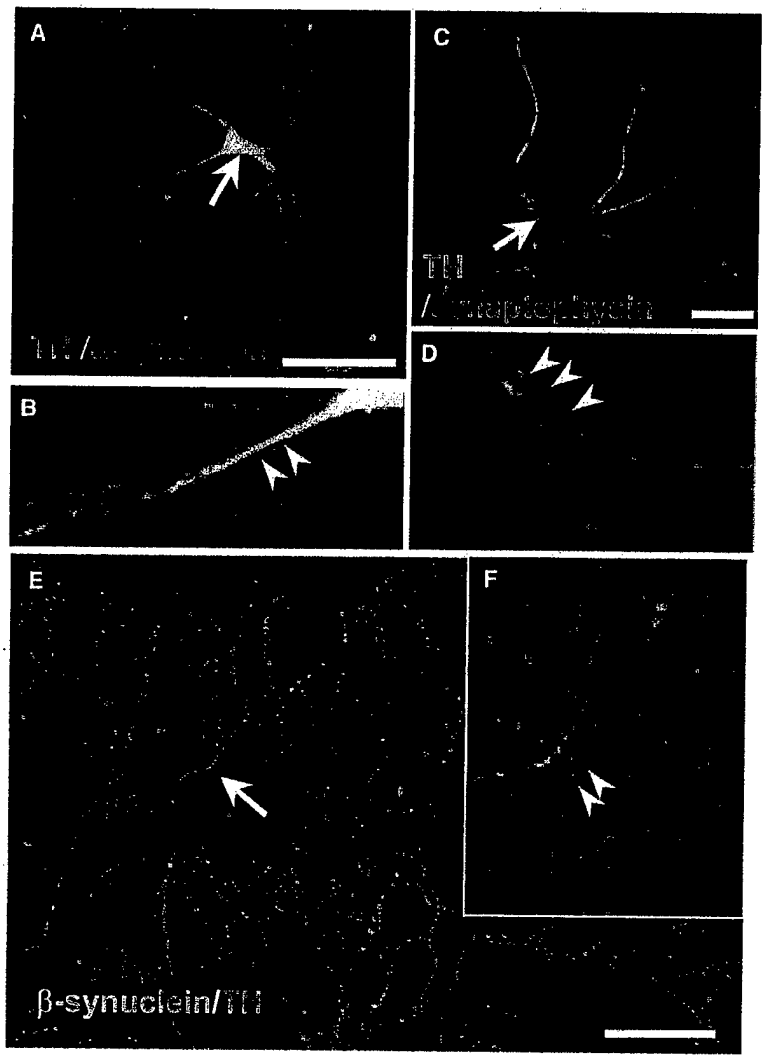


Figure 4. Characterization of α-Synuclein and β-Synuclein Expression in Primary Postnatal Midbrain Neurons

Primary neuronal cultures from postnatal mouse midbrain were stained with polyclonal antibodies to tyrosine hydroxylase (TH, green in [A]–[D]) and monoclonal antibodies to α-synuclein ([A] and [B] shows higher magnification) or synaptophysin ([C], higher magnification shown in [D]). The monoclonal antibody clone 42 (A and B) recognizes endogenous (mouse) α-synuclein. Staining for β-synuclein was performed using a polyclonal antibody (green in [E] and [F]) in combination with monoclonal anti-TH (red in [E] and [F]). All three proteins were expressed in both TH-positive (arrows) and TH-negative cells with similar patterns. Higher magnification of TH-positive cells (B, D, and F) demonstrated localization at the cell membrane, reminiscent of synaptic structures (arrowheads). All images were captured using a confocal microscope, and merged images of both channels are shown, yellow indicating overlap between the green and red channels. Scale bars represent 50 μm.

creased to 27% ± 1% in one clone and 30% ± 0.4% in the second. Consistent results were also obtained for two each of A53T and wild-type α-synuclein clones (data not shown). The differences between different clonal cell lines were significant ($p < 0.0001$) using two-way ANOVA, as was the effect of MG132 across all cell lines ($p < 0.0001$). We also exposed cells to the structurally unrelated inhibitor, lactacystin (Figure 1D). Lactacystin required concentrations of up to 25 μM to produce loss of cell viability to 70% (Figure 1D). Increased toxicity was noted in cell lines expressing either A30P or A53T α-synuclein. A small effect of wild-type α-synuclein was also seen. The differences between different clonal cell lines were significant ($p = 0.004$), as was the effect of lactacystin ($p < 0.0001$). Similar results were seen in experiments using a second set of clonal cell lines. Results presented here using MTT conversion are similar to previous reports using Trypan blue dye exclusion as a measure of cell death (Tanaka et al., 2001). To address the possibility that such effects might be due to inhibition of other proteases, we exposed cells to the cell-soluble calpain inhibitor E64d. This compound was without effect on cell viability up to 100 μM, which approached the limit of solubility (data not shown).

We next examined the mechanism by which mutant α-synuclein increases cellular sensitivity to proteasome inhibitors. We measured net proteasomal activity in living cells using the GFP^u reporter construct (Bence et al., 2001). When transfected into cells, the CL1 peptide (Gilon et al., 1998) sequence fused to GFP leads to rapid destruction of the protein, and in control cell lines we found only small amounts of GFP-CL1 peptide (Figure 2). The amount of reporter construct accumulation in cells transfected with wild-type α-synuclein was similar to that in vector-transfected cells but much higher in cells expressing A30P or A53T mutants (Figures 2A and 2B). The differences in amounts of GFP-CL1 between the cell lines were statistically significant ($p < 0.01$ by ANOVA). We also treated vector- or A30P-transfected cells with 5 μM lactacystin for 5 hr, which increased steady-state levels of the GFP-CL1 fusion protein. This effect was enhanced in the presence of A30P mutant α-synuclein (Figure 2D). Therefore, mutant α-synuclein inhibits proteasome function in a manner that is additive to the effect of pharmacological inhibition of the proteasome. There was no effect of lactacystin on steady-state α-synuclein levels in experiments where an increased GFP-CL1 reporter protein demonstrated unequivocally that

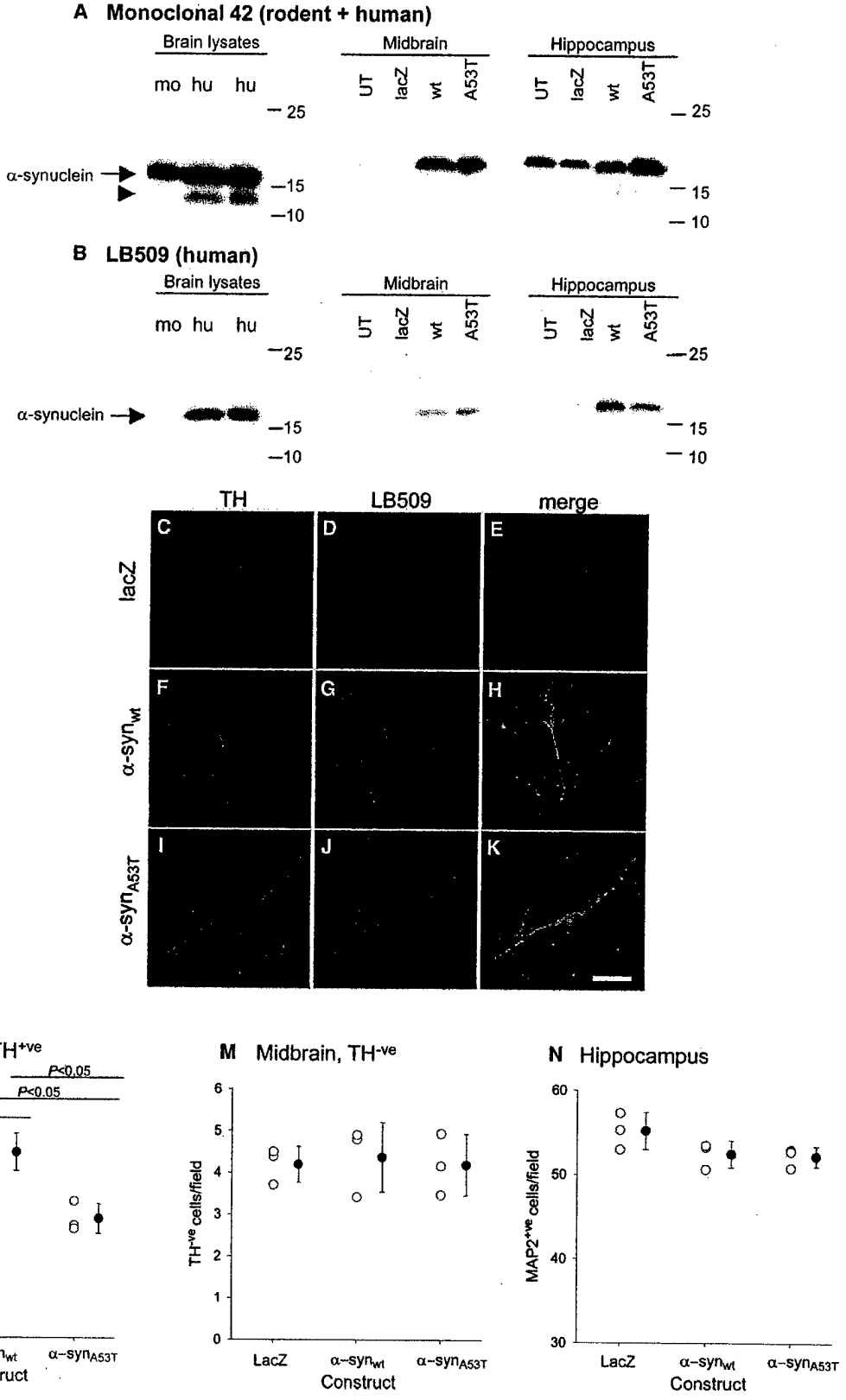


Figure 5. Overexpression of α-Synuclein in Primary Neurons

(A and B) Midbrain or hippocampal neurons were transduced with human α-synuclein (WT or A53T) and protein extracts (10 μg/lane) blotted with (A) either monoclonal 42 (recognizes rodent and human α-synuclein) or (B) LB509 (human α-synuclein only). Similar levels of expression were obtained with each of the viral constructs. Untransfected cells (UT) and cells transduced with a LacZ construct were used as controls.

there was proteasome inhibition (Figure 2E). Given the possible inhibitory effect of β-synuclein on α-synuclein toxicity (Hashimoto et al., 2001), we also examined β-synuclein expression in the same cell extracts. We were unable to detect β-synuclein protein in either basal conditions or after proteasome inhibition, although the antibodies used did detect expression of this protein in human brain (data not shown) and in primary mouse cultures (see below).

Manipulation of Parkin Expression Levels and Sensitivity to Proteasome Inhibitors

The above data demonstrate that the overexpression of mutant α-synuclein results in an increased sensitivity of cells to loss of viability induced by proteasome inhibition. We examined what effect increased parkin activity might have on the same parameters. Stable cell lines transfected with the parkin cDNA had substantially increased parkin protein expression (Figure 3A). Overexpression of parkin partially rescued cells from the toxic effects of MG132 (Figure 3B) or lactacystin (Figure 3C), with cell viability being 10%–20% higher in the parkin cell lines compared to controls at all doses of either compound. This effect was statistically robust ($p < 0.001$ for MG132, and $p < 0.004$ for lactacystin).

Further experiments confirmed that the observed rescue of parkin is dependent on its E3 ligase activity. A mutant form of parkin associated with loss of E3 ligase activity (R42P) was not able to rescue cells in an independent set of experiments (Figure 3D). Again, the differences between wild-type parkin and R42P parkin were statistically significant ($p < 0.001$ for differences between wild-type parkin and either vector alone or R42P). Coexpression of a dominant-negative E2 enzyme (UbCH7) also ameliorated the protective effect of parkin (Figure 3E). We also examined the effects of overexpression of a second E3 ligase, E6AP, which was not protective in these cells (Figure 3E). Experiments using the GFP^u construct demonstrated that there was no alteration in the amount of GFP-CL1 in the cells when transfected with parkin, either under basal conditions or after MG132 treatment (Figure 3F), although MG132 did increase GFP-CL1 levels in this experiment as in the experiments shown in Figure 2 . Parkin does not, therefore, act as an E3 ligase for this artificial proteasome reporter (see Discussion). However, the above experiments do not address what would occur if parkin activity within the cell was reduced, as occurs in ARJP. To model this effect, we generated cell lines with an antisense construct that had lower steady-state levels of parkin protein. This antisense knockdown of parkin does increase GFP^u levels within the cell, and MG132 has an additive effect (Figure 3F). This suggests that loss of parkin activity increases the level of heterologous substrates, presumably due to increased levels of parkin substrates competing for ubiquitination and/or proteasome-mediated degradation. Proteasome inhibitors are more toxic in cell lines transfected with an antisense parkin construct (Figure 3G).

Mutant α-Synuclein Triggers Selective Cell Death in Primary Neuronal Culture

Primary neuronal cultures from the midbrain of postnatal mice (Mena et al., 1997) were used to examine the effects of α-synuclein overexpression and proteasome inhibition on different neuronal groups. These cultures are predominantly from the nigral area and have a higher proportion of TH-positive catecholaminergic neurons than commonly used embryonic mesencephalic neuron preparations, with TH-positive cells representing about 20%–30% of the total neuronal population. The TH antibodies we used in these experiments recognized an appropriately sized band in extracts from whole mouse brain or from midbrain cultures but not in hippocampal cultures (data not shown). We characterized the expression of (mouse) α- and β-synuclein in these cells (Figure 4). Both TH-positive and TH-negative neurons expressed α-synuclein (Figures 4A and 4B) at the cell surface in a punctate pattern reminiscent of synaptic proteins such as synaptophysin (Figures 4C and 4D), similar to hippocampal neurons (Murphy et al., 2000). In addition, we also noted α-synuclein immunoreactivity in the cytoplasm. This was not limited to either TH-positive or TH-negative cells but was seen in many midbrain neurons. Expression of β-synuclein (Figures 4E and 4F) was also synaptic and seen in both cell populations at similar levels.

We next transduced primary midbrain cultures with wild-type or mutant α-synuclein and monitored expression by Western blotting or immunocytochemistry. Two monoclonal antibodies, clone 42 and LB509, were used to distinguish overexpressed human α-synuclein from endogenous mouse α-synuclein, respectively. Transduction with wild-type or mutant α-synuclein produced

---

Left-hand panels show controls using brain lysates from mouse (mo) or human (hu) cerebral cortex. Blotting using monoclonal antibody 42 yielded a major band at 19 kDa (arrow) plus a smaller degradation product with an apparent molecular weight of 16–17 kDa (arrowhead): this smaller product was not seen with LB509.

(C–K) Expression of human α-synuclein in catecholaminergic neurons. Primary midbrain catecholaminergic neurons (TH-positive, green) were transduced at multiplicities of infection (MOIs) of 5–10 with HSV1 expressing lacZ (C, D, and E) as a negative control or α-synuclein (WT, [F–H]; A53T, [I–K]). Transduction was demonstrated using a human specific monoclonal antibody LB509 (red), and more than 95% of cells were LB509 positive. Merged images are shown on the right of each set of photomicrographs. Scale bar in (K) represents 50 μm and applies to all panels. Representative data is shown from one of four experiments.

(L–M) Overexpression of mutant α-synuclein in primary midbrain neurons is associated with selective cell death of TH-positive cells. Cells were transduced with either LacZ (negative control), WT, or mutant (A53T) α-synuclein and cell numbers estimated by counting using TH and MAP2 staining. Although there was a significant reduction in TH-positive cell numbers (L), TH-negative cells in the midbrain (M) and hippocampal cells (N) were not affected. Each open circle is the average cell counts from one experiment with six fields counted in each of three cultures (hence n = 18): closed circles represent the mean from each of three experiments, with error bars representing the SEM between experiments. Statistical significance was assessed using one-way ANOVA with Student-Neuman-Kuells post-hoc tests between each group. ns, not significant.

similar levels of overexpression (Figures 5A and 5B). To confirm that TH-positive neurons in midbrain cultures were transduced, cells were costained for TH and human α-synuclein using LB509 (Figures 5C–5K). We estimated that after transduction at a multiplicity of infection (MOI) of 10, more than 95% of TH-positive neurons expressed human α-synuclein. TH-negative neurons were transduced at similarly high rates, showing almost 100% infectivity at MOIs of 5–10 (data not shown). Overexpression in this acute model does not result in the formation of microscopically visible α-synuclein aggregates (Figure 5J).

Cell counts were performed to assess whether the overexpressed α-synuclein induced any toxicity in TH-positive neurons. In each experiment, we counted six fields in each of three cultures. We also repeated the whole series three times with independent purifications of viral particles, and the data presented (Figure 5L) shows the interexperiment variation. We were able to demonstrate a clear toxic effect of A53T α-synuclein in TH-positive cells. We were not able to demonstrate an effect with wild-type α-synuclein under these conditions. TH-negative midbrain neurons (Figure 5M) or hippocampal neurons (Figure 5N) were unaffected by the presence of A53T α-synuclein.

TH-Positive Neurons Are Selectively Vulnerable to Proteasome Inhibition

We next examined whether proteasome inhibition was sufficient to produce selective neuronal cell loss in the same manner as α-synuclein overexpression. The numbers of TH-positive neurons in MG132 were decreased compared to controls, with remaining cells often showing shrinkage of cell bodies and retraction of neuritic processes (Figures 6A and 6B). Previous reports of cell death induced by proteasome inhibitors in the presence of mutant α-synuclein have given contradictory results on the mode of cell death, finding evidence for apoptosis (Tanaka et al., 2001) or autophagy (Stefanis et al., 2001). In our cultures exposed to proteasome inhibitors, counterstaining nuclei with Hoechst 33342 revealed that even in damaged cells the nuclei remained intact, unlike apoptosis. The numbers of TH-positive cells were significantly reduced at higher concentrations of lactacystin or MG132 (Figures 6C and 6E), while the number of TH-negative neurons was unaffected (Figures 6D and 6F). Using one-way ANOVA with Student-Newman-Kuells post-hoc test to evaluate the effects of proteasome inhibition on numbers of TH-positive cells remaining, MG132 had a significant ($p < 0.05$) effect at both 1 and 5 μM, whereas the effect of lactacystin was significant only at 10 μM. We also examined the effects of proteasome inhibitors on A53T α-synuclein-infected primary cultures (Figures 6G and 6H). The two treatments had an additive effect ($p < 0.01$ by ANOVA for all groups), although the loss of TH-positive cells was not complete after 24 hr, the time point used for this study. We examined cells transduced with A53T synuclein and treated with MG132 after staining with Hoechst 33342 (as in Figures 6A and 6B) and again did not find evidence for apoptosis (data not shown).

Parkin Rescues the Toxicity of Mutant α-Synuclein in Primary Neurons

We reasoned that as parkin protected cells against proteasome inhibition and mutant α-synuclein overexpression inhibited the proteasome then parkin might be protective against toxicity associated with overexpression of mutant α-synuclein. We repeated the experiments using A53T α-synuclein and coexpressed either lacZ as a control or parkin (Figures 7A and 7B). Cotransduction of parkin restored the number of counted neurons back to levels similar to cultures treated with lacZ alone. Using one-way ANOVA with post-hoc tests as above, the loss of TH-positive neurons was significantly different ($p < 0.05$) from controls and from cultures treated with parkin and α-synuclein ($p < 0.05$), but the difference between cotransduced cultures and control cells was not significant. Similarly to experiments in cell lines, Parkin was able to rescue to selective toxicity of MG132 to primary cells (Figures 7C and 7D).

Discussion

In the current study, we have examined manipulation of two genes that show association with familial PD on cellular sensitivity to proteasome inhibition and have examined aspects of neuronal selectivity. There was an increased sensitivity of cells overexpressing α-synuclein to proteasome inhibition, similar to previous reports (Lee et al., 2001b; Tanaka et al., 2001). Mutant α-synuclein also sensitizes cells to other insults (Junn and Mouradian, 2002; Ko et al., 2000; Lee et al., 2001b; Ostrerova-Golts et al., 2000; Zhou et al., 2000), but we and others (Stefanis et al., 2001; Tanaka et al., 2001) have demonstrated that one effect of mutant α-synuclein is to reduce the net proteasomal activity in living cells. Therefore, proteasome inhibition is likely to make a significant contribution to cell death induced by mutant α-synuclein. The inhibition of proteasome function by mutant α-synuclein may be a direct inhibition of proteasome activity, as α-synuclein can bind to one of the regulatory subunits of the proteasome (Ghee et al., 2000) or the presence of large amounts of misfolded or unfolded proteins, such as mutant α-synuclein, might inhibit the ubiquitin-proteasome pathway indirectly (Bence et al., 2001).

Overexpression of parkin protected against toxicity associated with reduced proteasome function. The lack of effect of the recessive R42P parkin mutation, which lacks ubiquitination activity (Shimura et al., 2001), demonstrates that mutant forms of parkin are unable to protect dopaminergic neurons against proteasome failure. Therefore, both genes implicated in familial PD alter the ability of neurons to tolerate reduced proteasome activity. We have shown that the E3 ligase activity of Parkin is required for protection, as a dominant-negative E2 mutant could ameliorate this effect. Experiments using the GFP^u reporter show that Parkin does not increase net proteasome activity, consistent with the role of this E3 ligase in controlling entry of target proteins into the proteasome via ubiquitination, and does not alter the steady-state levels of α-synuclein. Parkin is also protective in some other models of cell death, such as ER stress, but is not protective against all insults, including staurosporine (Imai et al., 2000). We suggest that parkin

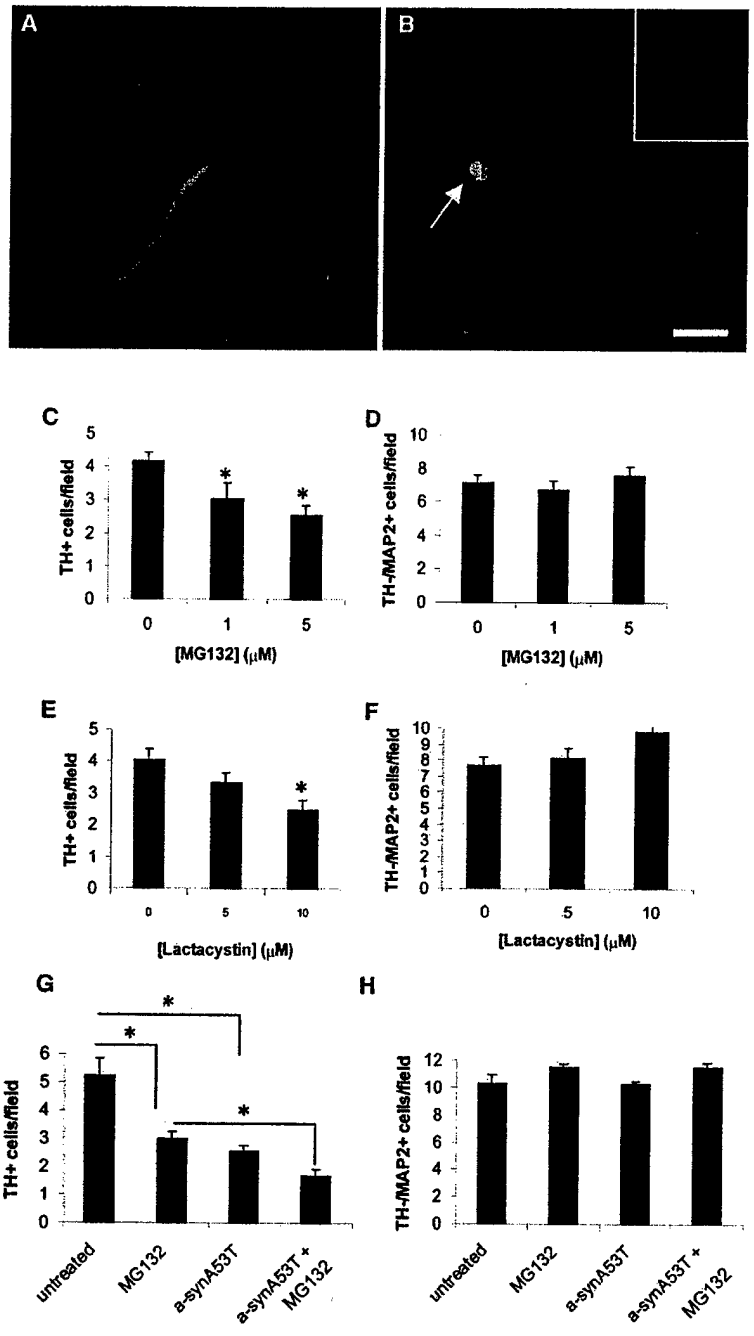


Figure 6. Catecholaminergic Neurons Are Preferentially Susceptible to Proteasome Inhibition in Primary Culture

(A and B) Primary midbrain cultures were left untreated (A) or exposed to 10 μM MG132 for 24 hr (B) and stained for MAP2 (red), TH (green), and Hoechst 33342 (blue) to demonstrate nuclear morphology. Exposure to the proteasome inhibitor caused TH-positive cells to become shrunken and retract processes, but nuclei remained intact (inset in [B]).

(C–F). Cell counts after exposure to MG132 (C and D) or lactacystin (E and F). Both inhibitors substantially affected the TH-positive neuronal population (C,E), whereas TH-negative/MAP2-positive neurons (D and F) were unaffected. Data are shown as cell counts from six randomly selected microscope fields in each of three replicate cultures (hence n = 18) and is representative of two to four independent experiments with different batches of primary cells. Statistical significance was assessed using one-way ANOVA with Student-Neuman-Kuells post-hoc tests between each group (*$p < 0.05$).

(G and H) Additive effect of α-synuclein overexpression and proteasome inhibition. Primary cells as above were transduced with mutant α-synuclein as in Figure 5 for 24 hr then exposed to MG132 for a further 24 hr. Cell counts revealed a loss of TH-positive neurons (G) after either treatment but an additive effect of both treatments together (*$p < 0.05$ by one-way ANOVA with Student-Newman-Kuells post-hoc test).

protects against the accumulation of its specific protein target(s). Accumulation of these downstream target proteins may also be promoted through ER stress due to a decreased ability of the cells for proper protein folding. The nature of the targets for parkin's E3 ligase activity is still under investigation, although several candidates have been reported (Chung et al., 2001; Imai et al., 2001; Shimura et al., 2001; Zhang et al., 2000). We also show that knockdown of parkin using a stable antisense construct increased sensitivity of cells to proteasome inhibition. This is, in many ways, a better model for loss-of-function mutations than overexpression of the wild-type protein. In ARJP, for example, homozygous large-scale deletions are predicted to reduce enzyme activity to extremely low levels and hence an antisense experiment is closer to this disease model than overexpression. In this experimental setting, there is a clear accumulation of heterologous substrates, as evidenced by accumulation of the GFP-CL1 reporter peptide, suggesting that loss-of-function alleles would decrease the ability of nigral neurons to regulate levels of proteasome substrates. It has been shown recently that proteasome inhibition in vivo damages nigral neurons (McNaught et al., 2002). Our results predict that loss of parkin function would have the same effect.

Both overexpression of mutant α-synuclein and pro-

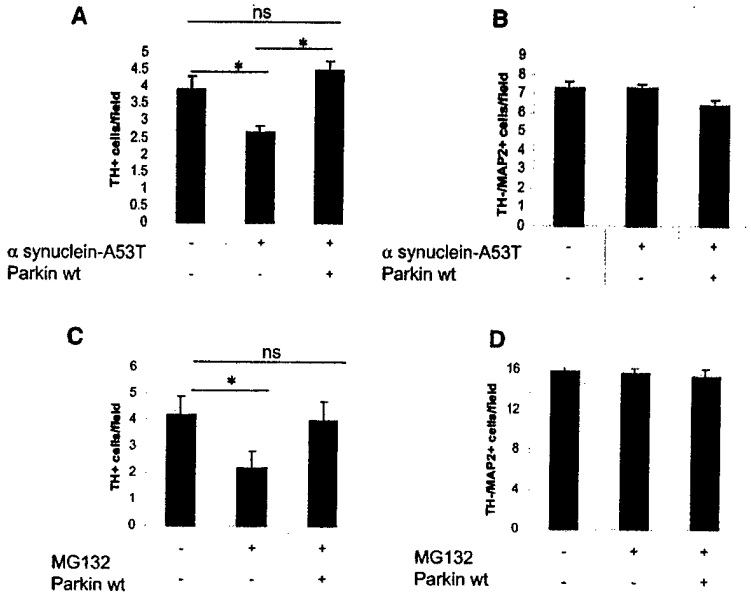


Figure 7. Parkin Rescues Toxicity Associated with Mutant α-Synuclein or Proteasome Inhibition in Primary Midbrain Cells

Primary midbrain neurons were transduced with both mutant α-synuclein and wild-type human parkin. Where either virus was absent (−), LacZ was substituted to keep the total number of viral particles similar. There was a significant reduction in the number of TH-positive neurons (A), which was ameliorated by coexpressing parkin. No effect on TH-negative cells was noted (B). Data are shown as cell counts from six randomly selected microscope fields in each of three replicate cultures (hence n = 18), and statistical significance was assessed using one-way ANOVA with Student-Neuman-Kuells post-hoc tests between each group (*$p < 0.05$; ns, not significant). Similar protective effects were noted using exposure to MG132 to induce toxicity (C and D) where selective toxicity to TH-positive cells was noted (C). The numbers of cell counts and statistical tests are as in (A) and (B).

teasome inhibition produce selective loss of TH-positive neurons. Therefore, proteasome inhibition is sufficient to mimic the effects of mutant α-synuclein. It is not the case that all catecholaminergic neurons are severely affected in PD, while all non-dopaminergic neurons are spared (Braak and Braak, 2000). Nonetheless, we have shown that the SN contains a population of TH-positive cells that are particularly sensitive to both proteasome inhibition and α-synuclein overexpression. The higher sensitivity of catecholaminergic neurons to damage induced by overexpression of mutant α-synuclein may be due to the ability of catecholamines to promote the formation of protofibrillar forms of α-synuclein (Conway et al., 2001). It has been suggested that α-synuclein protofibrils are a major toxic species of this protein, and mutations in α-synuclein also promote protofibril formation (Conway et al., 2000). Recently, it has been shown that, in cultured human dopaminergic neurons, dopamine is required for the toxic effects of mutant α-synuclein, supporting this hypothesis (Xu et al., 2002). We have not seen a protective effect of the same tyrosine hydroxylase inhibitor (α-methyl-*para*-tyrosine; AMPT) in the stable cell lines we used in this study, but this is confounded by a small toxic effect of AMPT alone in our hands (M.R.C. and M.B., unpublished data). In our experiments, mutant α-synuclein is more toxic to TH-positive cells than the wild-type protein. Overexpression of α-synuclein in embryonic mesencephalic cultures (Zhou et al., 2000) or human dopaminergic cells (Xu et al., 2002) produces a similar effect. In some mouse transgenic models, there is pathology associated with a substantial overexpression of wild-type α-synuclein, and there are reports of dopaminergic cell loss in some (Masliah et al., 2000) but not all (Matsuoka et al., 2001; Rathke-Hartlieb et al., 2001; VanDerPutten et al., 2000) models. In a *Drosophila* model, loss of dopaminergic neurons is also seen (Feany and Bender, 2000) with mutant α-synuclein having a more substantial effect than wild-type. Our data are similar to viral-mediated gene transfer experiments where mutant α-synuclein produces nigral cell loss (Kirik et al., 2002).

We did not find microscopically visible protein aggregates, nor did we see the formation of higher molecular weight species of α-synuclein as seen in some models (Lee et al., 2001a). We believe that this is compatible with the idea that protein aggregation to the extent of formation of insoluble fibrillar species is not a required step for α-synuclein toxicity. Soluble protein complexes appear to mediate the toxic effects of mutant α-synuclein in human dopaminergic cells (Xu et al., 2002). The formation of protein aggregates is clearly relevant to the human disease, as the formation of insoluble protein deposits in the form of Lewy bodies occurs in surviving neurons. Previous results using iron/dopamine-mediated toxicity have shown that although formation of protein aggregates and toxicity can be seen under similar conditions, these are dissociatable phenomena (Ostrerova-Golts et al., 2000). Proteasome inhibition might conceivably affect the fibrillization properties of α-synuclein through the phenomenon of "molecular crowding" (Ellis, 2001). Two recent studies have demonstrated that increasing the concentration of macromolecules in the immediate surroundings of α-synuclein increases its propensity to form protofibrillar and fibrillar species (Shtilerman et al., 2002; Uversky et al., 2002). By inhibiting proteasome function, concentrations of many cytosolic proteins will increase, thereby inducing a molecular crowding effect. Therefore, if formation of protofibrillar forms of α-synuclein is important for the toxicity of the mutant forms, proteasome inhibitors are likely to accelerate this process, without having an effect on net α-synuclein protein concentrations. Protection by parkin of TH-positive neurons exposed to either mutant α-synuclein or proteasome inhibition suggests that these two stresses damage cells by similar mechanisms. In the results reported here, antisense-mediated knockdown of parkin also increased levels of heterologous substrates, again potentially inducing accumulation of several toxic proteins and inducing a crowding effect.

The protective effect of parkin on loss of TH-positive neurons mediated by mutant α-synuclein demonstrates that these two proteins have interrelated effects. The

above data suggest that an increased sensitivity of cells to the toxic effects of proteasome inhibition link α-synuclein and parkin as well as providing an explanation for the selective loss of a subgroup of dopaminergic neurons in PD. Whether proteasome inhibition will provide a full explanation for neuronal damage in PD is not clear. There are several genes linked to familial PD that remain to be identified, and it will be critical to evaluate whether manipulations of these products also produces increased sensitivity to proteasome inhibition.

Experimental Procedures

Human Neuroblastoma Cell Lines

The production of stable cell lines overexpressing wild-type or mutant α-synuclein from parental BE (2)-M17 human dopaminergic neuroblastoma cells has been detailed elsewhere (Ostrerova-Golts et al., 2000). Full-length parkin cDNA was cloned into the same vector and transfections performed as described previously (Ostrerova-Golts et al., 2000). An antisense construct was made by placing the first 100 bp of coding sequence for parkin into pCDNA3.1 in the reverse orientation relative to the vector promoter. Stable clones were prepared as above and screened for reduced parkin expression. For the present study, clonal lines for both α-synuclein and parkin were made by limiting dilution and were maintained on 500 μg mL^{-1} G418. Stable clonal lines were screened for α-synuclein expression by Western blotting using monoclonal antibody to α-synuclein (Clone 42, Transduction Labs). Cell lysates (10 μg total protein per lane) were separated on 16% SDS-PAGE gels (Novex) and transferred to Immobilon membranes (Immobilon, Inc). A soluble extract of adult human cerebral cortex was used as a positive control. After probing with primary antibody (1:1000), blots were developed with peroxidase-labeled secondary antibodies (Jackson Immunochemicals) using enhanced chemiluminescence substrates (Amersham). Blots were reprobed with β-actin (Sigma, clone AC15, 1:5000) to verify equal loading. Quantitation of α-synuclein expression was performed by capturing enhanced chemiluminescence using a CCD camera-based system (AlphaImager, Alpha Innotech Corp). Parkin expression was also monitored by Western blotting using a rabbit polyclonal antibody to the C terminus of parkin (Cell Signaling Technology, 1:2000 dilution). Cell viability was assessed using the MTT assay, as described previously (Cookson et al., 1998). For each experiment, 8 wells were used per concentration of either compound, and each experiment was repeated three times with similar results.

Measurement of Proteasome Function in Living Cells

Stable cell lines were transiently transfected with the GFP^u construct (Bence et al., 2001) and 24 hr later were treated with 5 μM lactacystin for 6 hr or left untreated as controls. Protein extracts and Western blotting were performed as above to measure steady-state amounts of GFP-CL1 fusion protein in the cells using a monoclonal antibody to GFP (Clontech). Blots were reprobed with monoclonal anti-α-synuclein and subsequently with monoclonal anti β-actin as described above.

Primary Cell Cultures

Primary cell cultures were prepared from postnatal mouse midbrain using methods described by Burke et al. (Burke et al., 1998). Briefly, midbrains containing SN and ventral tegmental area were dissected from 2-day-old postnatal mouse pups, using anatomical landmarks as described (Burke et al., 1998; Mena et al., 1997). Neurons from these areas were dissociated with papain and plated on top of preestablished cortical glia cell monolayers at a density of 80,000 cells per well in growth medium which had been preconditioned by adding to glial feeder layers 24 hr prior to plating neurons. Neurons from 3- to 4-week cultures were exposed for 24 hr to MG132 or lactacystin (Calbiochem) and toxicity assessed by staining for tyrosine hydroxylase (TH) and microtubule-associated protein 2 (MAP2). Cells were fixed and permeabilized with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% (w/v) saponin. After quenching (0.1 M glycine in DPBS, 20 min) and blocking (5% v/v goat serum plus 5% FBS in DPBS, 30 min), primary antibodies were applied overnight at 4°C. These were a combination of polyclonal antibody to TH (Chemicon, 1:200) and monoclonal anti-MAP2 (clone AP-20, Sigma) both at a dilution of 1:200. Secondary antibodies were goat anti-rabbit conjugated to Alexafluor488 (Molecular Probes) and goat anti-mouse conjugated to AlexaFluor 568. For estimations of cell numbers, six random microscope fields (using a 20× objective) were counted in each of three sister cultures, for a total of 18 fields per condition, counting all neuronal cells within each field. Each experiment was repeated two to four times with different batches of primary cells. For staining of α-synuclein, monoclonal anti-human α-synuclein LB509 (Zymed) or monoclonal anti-rodent α-synuclein (clone 42, listed above) were used in the above protocol at a dilution of 1:200. A polyclonal antibody to β-synuclein (Chemicon) was used at 1:500 in conjunction with monoclonal antibody to TH (also Chemicon, 1:200).

Viral Transduction

α-synuclein or parkin cDNAs were cloned into pHSVPrpUC and packaged into recombinant viral particles using 5dl1.2 helper virus and the 2-2 packaging cell line as described (Neve et al., 1997) and purified using sucrose gradients. A control virus expressing LacZ (from pHSVlacZ, Coopersmith and Neve, 1999) was prepared at the same time. Recombinant viruses were titred on human neuroblastoma cell lines. Primary cells were transduced with viral particles at a multiplicity of infection (MOI) of 10. In a small series of experiments, we extracted cultures grown in 6-well plates and blotted for α-synuclein as above.

Statistical Analyses

Differences in the responses of cell lines to proteasome inhibitors were evaluated using two-way analysis of variance (ANOVA) with cell line and concentration of each proteasome inhibitor as variables. For primary cell counts, one-way ANOVA with Student-Neuman-Kuells post-hoc test was used to assess differences between treatments with proteasome inhibitors or with transduction with different viral constructs. For each of these experiments, six fields were counted in each of three independent cultures, hence $n = 18$. In experiments comparing the toxicity associated with overexpression of α-synuclein, we repeated this whole set of experiments three times.

Acknowledgments

The authors would like to thank Dr. David Sulzer, Columbia University, for sharing the methods for culturing postnatal mouse midbrain neurons. We would also like to acknowledge the generous gifts of reagents for HSV-1 packaging from Dr. Rachael Neve, Harvard Medical School, Boston, and Neil Bence and Dr. Ron Kopito of Stanford University for the gift of the GFP^u plasmid. Dr. Ted Dawson, Johns Hopkins Medical Institute, kindly provided the dominant-negative UbCH7 construct. This work was supported by NINDS grants P01-NS40256 and RO1-NS41816-01.

Received: January 16, 2002
Revised: November 21, 2002

References

Ancolio, K., Alves da Costa, C., Ueda, K., and Checler, F. (2000). Alpha-synuclein and the Parkinson's disease-related mutant Ala53Thr-alpha-synuclein do not undergo proteasomal degradation in HEK293 and neuronal cells. Neurosci. Lett. *285*, 79–82.

Bence, N.F., Sampat, R.M., and Kopito, R.R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. Science *292*, 1552–1555.

Bennett, M.C., Bishop, J.F., Leng, Y., Chock, P.B., Chase, T.N., and Mouradian, M.M. (1999). Degradation of alpha-synuclein by proteasome. J. Biol. Chem. *274*, 33855–33858.

Braak, H., and Braak, E. (2000). Pathoanatomy of Parkinson's disease. J. Neurol. Suppl. *247*, 3–10.

Burke, R.E., Antonelli, M., and Sulzer, D. (1998). Glial cell line-derived

neurotrophic growth factor inhibits apoptotic death of postnatal substantia nigra dopamine neurons in primary culture. J. Neurochem. *71*, 517–525.

Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L., and Dawson, T.M. (2001). Parkin ubiquitinates the α-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat. Med. *7*, 1144–1150.

Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., and Lansbury, P.T. (2000). Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. Proc. Natl. Acad. Sci. USA *97*, 571–576.

Conway, K.A., Rochet, J.-C., Bieganski, R.M., and Lansbury, P.T. (2001). Kinetic stabilization of the α-synuclein protofibril by a dopamine-α-synuclein adduct. Science *294*, 1346–1349.

Cookson, M.R., Ince, P.G., and Shaw, P.J. (1998). Peroxynitrite and hydrogen peroxide induced cell death in the NSC34 neuroblastoma x spinal cord cell line: Role of poly(ADP-ribose) polymerase. J. Neurochem. *70*, 501–508.

Coopersmith, R., and Neve, R.L. (1999). Expression of multiple proteins within single primary cortical neurons using a replication deficient HSV vector. Biotechniques *27*, 1156–1160.

Ellis, R.J. (2001). Macromolecular crowding: an important but neglected aspect of the intracellular environment. Curr. Opin. Struct. Biol. *11*, 114–119.

Farrer, M., Destee, T., Becquet, E., Wavrant-De Vrieze, F., Mouroux, V., Richard, F., Defebvre, L., Lincoln, S., Hardy, J., Amouyel, P., and Chartier-Harlin, M. (2000). Linkage exclusion in French families with probable Parkinson's disease. Mov. Disord. *15*, 1075–1083.

Farrer, M., Chan, P., Chen, R., Tan, L., Lincoln, S., Hernandez, D., Forno, L., Gwinn-Hardy, K., Petrucelli, L., Hussey, J., et al. (2001). Lewy bodies and parkinsonism in families with parkin mutations. Ann. Neurol. *50*, 293–300.

Feany, M.B., and Bender, W.W. (2000). A *Drosophila* model of Parkinson's disease. Nature *404*, 394–398.

Ghee, M., Fournier, A., and Mallet, J. (2000). Rat alpha-synuclein interacts with tat binding protein *1*, a component of the 26S proteasomal complex. J. Neurochem. *75*, 2221–2224.

Gilon, T., Chomsky, O., and Kulka, R.G. (1998). Degradation signals for ubiquitin system proteolysis in Saccharomyces cerevisiae. EMBO J. *17*, 2759–2766.

Hashimoto, M., Rockenstein, E., Mante, M., Mallory, M., and Masliah, E. (2001). beta-Synuclein inhibits alpha-synuclein aggregation: a possible role as an anti-parkinsonian factor. Neuron *32*, 213–223.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. Annu. Rev. Biochem. *67*, 425–479.

Imai, Y., Soda, M., and Takahashi, R. (2000). Parkin supresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. J. Biol. Chem. *275*, 35661–35664.

Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. (2001). An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell *105*, 891–902.

Junn, E., and Mouradian, M.M. (2002). Human a-synuclein overexpression increases intracellular reactive oxygen species levels and susceptibility to dopamine. Neurosci. Lett. *320*, 146–150.

Kirik, D., Rosenblad, C., Burger, C., Lundberg, C., Johansen, T.E., Muzyczka, N., Mandel, R.J., and Bjorklund, A. (2002). Parkinson-like neurodegeneration induced by the targeted overexpression of α-synuclein in the nigrostriatal system. J. Neurosci. *22*, 2780–2791.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature *392*, 605–608.

Ko, L., Mehta, N.D., Farrer, M., Easson, C., Hussey, J., Yen, S., Hardy, J., and Yen, S.H. (2000). Sensitization of neuronal cells to oxidative stress with mutated human alpha-synuclein. J. Neurochem. *75*, 2546–2554.

Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J.T., Schols, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat. Genet. *18*, 106–108.

Lee, H.J., Shin, S.Y., Choi, C., Lee, Y.H., and Lee, S.J. (2001a). Formation and removal of alpha-synuclein aggregates in cells exposed to mitochondrial inhibitors. J. Biol. Chem. *277*, 5411–5417.

Lee, M., Hyun, D., Halliwell, B., and Jenner, P. (2001b). Effect of the overexpression of wild-type or mutant alpha-synuclein on cell susceptibility to insult. J. Neurochem. *76*, 998–1009.

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., et al. (1998). The ubiquitin pathway in Parkinson's disease. Nature *395*, 451–452.

Maraganore, D.M., Farrer, M.J., Hardy, J.A., Lincoln, S.J., McDonnell, S.K., and Rocca, W.A. (1999). Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. Neurology *53*, 1858–1860.

Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000). Dopaminergic loss and inclusion body formation in α-synuclein mice: implications for neurodegenerative disorders. Science *287*, 1265–1269.

Matsuoka, Y., Vila, M., Lincoln, S., McCormack, A., Picciano, M., LaFrancois, J., Yu, X., Dickson, D., Langston, W.J., McGowan, E., et al. (2001). Lack of nigral pathology in transgenic mice expressing human alpha-synuclein driven by the tyrosine hydroxylase promoter. Neurobiol. Dis. *8*, 535–539.

McNaught, K.S., and Jenner, P. (2001). Proteasomal function is impaired in substantia nigra in Parkinson's disease. Neurosci. Lett. *297*, 191–194.

McNaught, K.S., Bjorklund, L.M., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C.W. (2002). Proteasome inhibition causes nigral degeneration with inclusion bodies in rats. Neuroreport *13*, 1437–1441.

Mena, M.A., Khan, U., Togasaki, D.M., Sulzer, D., Epstein, C.J., and Przedborski, S. (1997). Effects of wild-type and mutated copper/zinc superoxide dismutase on neuronal survival and L-DOPA-induced toxicity in postnatal midbrain culture. J. Neurochem. *69*, 21–33.

Murphy, D.D., Rueter, S.M., Trojanowski, J.Q., and Lee, V.M. (2000). Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. J. Neurosci. *20*, 3214–3220.

Neve, R.L., Howe, J.R., Hong, S., and Kalb, R.G. (1997). Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. Neuroscience *79*, 435–447.

Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J.M., Farrer, M., and Wolozin, B. (2000). The A53T α-synuclein mutation increases iron-dependent aggregation and toxicity. J. Neurosci. *20*, 6048–6054.

Pakkenberg, B., Moller, A., Gundersen, H.J., Mouritzen Dam, A., and Pakkenberg, H. (1991). The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. J. Neurol. Neurosurg. Psychiatry *54*, 30–33.

Paxinou, E., Chen, Q., Weisse, M., Giasson, B.I., Norris, E.H., Rueter, S.M., Trojanowski, J.Q., Lee, V.M., and Ischiropoulos, H. (2001). Induction of alpha-synuclein aggregation by intracellular nitrative insult. J. Neurosci. *21*, 8053–8061.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., et al. (1997). Mutation in the α-synuclein gene identified in families with Parkinson's disease. Science *276*, 2045–2047.

Rathke-Hartlieb, S., Kahle, P.J., Neumann, M., Ozmen, L., Haid, S., Okochi, M., Haass, C., and Schulz, J.B. (2001). Sensitivity to MPTP is not increased in Parkinson's disease-associated mutant alpha-synuclein transgenic mice. J. Neurochem. *77*, 1181–1184.

Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minoshima, S., Shimuzu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki,

T. (2000). Familial parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat. Genet. *25*, 302–305.

Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S., and Selkoe, D.J. (2001). Ubiquitination of a new form of α-synuclein by parkin from human brain: implications for Parkinson's disease. Science *293*, 263–269.

Shtilerman, M.D., Ding, T.T., and Lansbury, P.T. (2002). Molecular crowding accelerates fibrillization of alpha-synuclein: could an increase in the cytoplasmic protein concentration induce Parkinson's disease? Biochemistry *41*, 3855–3860.

Stefanis, L., Larsen, K.E., Rideout, H.J., Sulzer, D., and Greene, L.A. (2001). Expression of A53T mutant but not wild-type α-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. J. Neurosci. *21*, 9549–9560.

Tanaka, Y., Engelender, S., Igarashi, S., Rao, R.K., Wanner, T., Tanzi, R.E., Sawa, A.L., Dawson, V., Dawson, T.M., and Ross, C.A. (2001). Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. Hum. Mol. Genet. *10*, 919–926.

Tofaris, G.K., Layfield, R., and Spillantini, M.G. (2001). α-Synuclein metabolism and aggregation is linked to ubiquitin-independent degradation by the proteasome. FEBS Lett. *509*, 22–26.

Uversky, V.N., Cooper, E.M., Bower, K.S., Li, J., and Fink, A.L. (2002). Accelerated α-synuclein fibrillation in crowded milieu. FEBS Lett. *515*, 99–103.

VanDerPutten, H., Widerhold, K.-H., Probst, A., Barbieri, S., Misti, C., Danner, S., Kauffmann, S., Hofele, K., Spooren, W.P., Ruegg, M.A., et al. (2000). Neuropathology in mice expressing human α-synuclein. J. Neurosci. *20*, 6021–6029.

Wintermeyer, P., Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Berg, D., Becker, G., Leroy, E., Polymeropoulos, M., Berger, K., et al. (2000). Mutation analysis and association studies of the UCHL1 gene in German Parkinson's disease patients. Neuroreport *11*, 2079–2082.

Xu, J., Kao, S.-Y., Lee, F.J.S., Song, W., Jin, L.-W., and Yankner, B.A. (2002). Dopamine-dependent neurotoxicity of α-synuclein: a mechanism for selective neurodegeneration in Parkinson disease. Nat. Med. *8*, 600–606.

Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L., and Dawson, T.M. (2000). Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc. Natl. Acad. Sci. USA *97*, 13354–13359.

Zhou, W., Hurlbert, M.S., Schaack, J., Prasad, K.N., and Freed, C.R. (2000). Overexpression of human alpha-synuclein causes dopamine neuron death in rat primary culture and immortalized mesencephalon-derived cells. Brain Res. *866*, 33–43.

Appendix 5

Tau phosphorylation increases in symptomatic mice over-expressing A30P α-synuclein

[1]Frasier, M., [1,2]Walzer, M., [1]McCarthy, L., [2]Magnuson, D., [2]Lee, J.M., [3]Haas, C., [3]Kahle, P. and [1*]Wolozin, B.

Dept. of [1]Pharmacology and [2]Pathology, Loyola University Medical Center, Maywood, IL; [3]University of Munich, Germany

*Corresponding author

Address Correspondence to:

Benjamin Wolozin, M.D., Ph.D.

Dept. of Pharmacology

Loyola University Medical Ctr.

Bldg. 102/3634, 2160 S. 1st Ave.

Maywood, IL 60153

708-216-6195 (Phone)

708-216-6596 (FAX)

bwolozi@lumc.edu

Abbreviations: cyclin dependent kinase 5 (CDK5); glial fibrillary acidic protein (GFAP); glycogen sythase kinase –3β (GSK-3 β); Parkinson's disease (PD)

Acknowledgements: We would like to thank Nancy Muma (Loyola) for her useful suggestions.

This research was supported by grants from NINDS (NS41786-01), NIA (Ag17485) and USAMRC (DAMD17-01-1-0781).

ABSTRACT

Mice over-expressing mutant α-synuclein develop a progressive loss of motor function associated with the accumulation of aggregated α-synuclein in neurons of the brain stem. We also observed the concurrent accumulation of ubiquitin and wide-spread astrocytosis throughout the brain stem region. Recent reports suggest that tau pathology might also be associated with Parkinson's disease and aggregation of α-synuclein. We now report that mice over-expressing A30P α-synuclein develop abnormally phosphorylated tau as they accumulate aggregated α-synuclein. The increased phosphorylation of tau occurs at S396/404 and S202 as shown by immunoblotting and immunocytochemical studies with the PHF-1 and AT8 antibodies, respectively. Immunoblotting studies show that the phosphorylated tau is present only in symptomatic mice that also show abundant aggregated α-synuclein. The neurons that show the most abundant staining for phosphorylated tau and aggregated α-synuclein occur in spatially distinct areas throughout the reticular formation in the brain stem. Approximately 47 ± 11% of neurons positive for phosphorylated tau show punctate α-synuclein reactivity, suggesting that small aggregates of α-synuclein might precipitate the changes in tau. Cyclin dependent kinase 5 is known to phosphorylate tau at S396/404 and S202, and immunocytochemical studies demonstrate co-localization of abnormally phosphorylated tau with activated cyclin dependent kinase 5. These results suggest that α-synuclein pathology can directly lead to early pathological changes in tau.

INTRODUCTION

The protein α-synuclein appears to play an important role in the pathophysiology of Parkinson disease (PD). Lewy bodies, a pathological hallmark of PD, are composed primarily of the proteins α-synuclein, and mutations in α-synuclein at A53T and A30P are associated with familial PD (Polymeropoulos et al., 1997; Kruger et al., 1998; Spillantini et al., 1998). α-synuclein mutations (A53T and A30P) associated with familial PD both increase the tendency of α-synuclein to aggregate spontaneously and in response to exogenous factors, such as metals and oxidative stress (Polymeropoulos et al., 1997; Kruger et al., 1998; Hashimoto et al., 1999; Paik et al., 1999; Ostrerova-Golts et al., 2000; Paik et al., 2000). The A53T and A30P mutations in α-synuclein also cause age-dependent α-synuclein aggregation and neuronal injury in transgenic mice and Drosophila (Feany and Bender, 2000; Masliah et al., 2000; Kahle et al., 2001; Giasson et al., 2002; Lee et al., 2002). These results provide support for the putative role of α-synuclein aggregation in neurodegeneration.

The function of α-synuclein protein is incompletely understood. The α-synuclein protein contains regions of homology with the protein chaperone 14-3-3, displays functions resembling a chaperone (Ostrerova et al., 1999; Souza et al., 2000), and also binds many proteins including 14-3-3, protein kinase C, phospholipase C δ, extracellular regulated kinase, synphilin-1, tyrosine hydroxylase, the dopamine transporter, SEPT4 and the S6' proteasomal protein (Jenco et al., 1998; Engelender et al., 1999; Ostrerova et al., 1999; Pronin et al., 2000; Choi et al., 2001; Lee et al., 2001; Sharon et al., 2001; Ihara et al., 2003). Functional studies suggest that α-synuclein plays a role in vesicular exocytosis (Murphy et al., 2000). However, the relationship between α-synuclein function and its role in neurodegeneration remain unclear.

The microtubule associated protein tau is another protein implicated in the pathophysiology of many neurodegenerative diseases. Increasing evidence suggests overlap between disorders showing tau and α-synuclein aggregation. Tau and α-synuclein pathology both occur in Alzheimer's disease, PD, Guam-Parkinson-ALS dementia complex, and PD caused by mutations α-synuclein (Duda et al., 2002; Forman et al., 2002; Ishizawa et al., 2003). Abnormal phosphorylated tau is present in Lewy bodies found in sporadic PD patients, and occurs in neurons near areas containing α-synuclein pathology (Ishizawa et al., 2003). *In vitro*

evidence also links α-synuclein and tau as α-synuclein binds tau *in vitro*, and stimulates tau phosphorylation by protein kinase A *in vitro* (Jensen et al., 1999; Giasson et al., 2003). Recent results indicate that α-synuclein enhances tau fibrillization *in vitro*, and that abnormal tau fibrils are present in the brains of symptomatic transgenic mice over-expressing mutant A53T synuclein (Giasson et al., 2003).

Therefore, in order to investigate how mutant A30P α-synuclein protein affects tau pathology, we examined the effects of α-synuclein on tau phosphorylation in transgenic mice over-expressing A30P α-synuclein (Kahle et al., 2001). We report that symptomatic A30P α-synuclein transgenic mice exhibit abnormal tau phosphorylation associated with increased activity of cyclin dependent kinase 5.

MATERIALS AND METHODS

Animals

The human [A30P]α-synuclein transgene had been injected in hybrid B6 / DBA oocytes (Kahle et al. 2000). Founders were extensively (7-10 generations) back-crossed into a C57Bl/6 background. Intercrossing of the highest expressing line 31 yielded a stable colony of homozygous 31H mice (Neumann et al. 2002). These were the animals used in the present study. The mice develop symptoms between 6-14 months. The symptoms begin with a tremor and progress to an end-stage phenotype characterized by muscular rigidity, postural instability and ultimately paralysis. Mice that progressed to end stage symptoms were sacrificed by cervical dislocation and their brains hemi-sectioned. The right hemisphere was fixed in formalin and subsequently embedded in paraffin for immunohistochemistry. The left hemisphere was flash frozen in methyl-2-butane and kept at -80°C for immunoblot analysis.

Antibodies and Immunohistochemistry

Brains were sagitally sectioned at a thickness of 4 μm and mounted on Superfrost-plus slides (Fisher Scientific). Distinct monoclonal phospho-specific tau antibodies AT8 (Pierce Endogen, 1:200, Ser 199, 202, Thr 205) and PHF-1 (generously provided by P. Davies, 1:200, Ser 396, 404) were used to detect phosphorylated tau epitopes. Antibodies against α-synuclein included a mouse monoclonal anti-α-synuclein antibody directed against the N-terminus (Zymed, 1:100)

and a rabbit polyclonal antibody directed against amino acids 116-131 (used for immunohistochemistry, 1:500) (Ostrerova-Golts et al., 2000). Other antibodies used were: anti-glial fibrillary acidic protein (Dako, 1:500), rabbit polyclonal anti-activated CDK5 antibody (Santa Cruz Biotech, 1:50) and rabbit polyclonal anti-ubiquitin antibody (Dako, 1:500). Immunofluorescence was performed using the same antibody concentrations as above. For immunofluorescence, sections were first treated with 70% formic acid at room temperature for 15 minutes, rinsed in PBS for 10 minutes, and then blocked for 20 minutes in 2% fetal bovine serum and 1% normal goat serum in PBS at room temperature. Primary antibody was incubated on the sections overnight at 4°C and followed by two ten minute washes in PBS. Fluorescent cy2 anti-mouse (1:2500) and rhodamine anti-rabbit (1:200) secondary antibodies (Jackson Immunology) were incubated on the sections for 1 hour at room temperature in the dark. Following two more 10 minute washes in PBS, coverslips were applied using the Fluormount G mounting media (Electron Microscopy Sciences, Washington PA).

Brain Tissue Extraction

Brain tissue extraction was performed as previously described by Sahara et al. (Sahara et al., 2002) with minor modifications. Left hemi-brains were weighed and homogenized in 3 volumes of TBS (TBS, pH 7.4, 1 mM EDTA, 5mM sodium pyrophosphate, 30 mM glycerol 2-phosphate, 30 mM sodium fluoride, 1 mM EDTA) containing a protease inhibitor cocktail (Sigma-Aldrich). Protein content was determined via the BCA method and total protein and concentration was adjusted with the homogenization buffer to be equal between samples. Samples were then centrifuged at 150,000 *g* for 15 min at 4°C in a Beckman TLA 1004 rotor (Beckman, Palo Alto, CA). The resulting pellet was re-homogenized in three volumes of a salt sucrose buffer (10 mM Tris-HCL, pH 7.4, 0.8 M NaCl, 10% sucrose, 1 mM EDTA) with a protease inhibitor cocktail added immediately before use while the supernatant was labeled as S1 and frozen at -80°C. The resuspended pellet was again centrifuged at 150,000 *g* for 15 min at 4°C, this time, the pellet was discarded and the supernatant was brought to a 1% sarkosyl solution and incubated at 37°C for 1 hour with gentle shaking. After centrifugation (150,000 *g* for 30 min at 4°C) the supernatant was kept, labeled as S2, and frozen at -80°C. The pellet was re-suspended in Tris/EDTA (10 mM Tris HCL ,pH 7.4, 1mM EDTA), washed once more with 1% sarkosyl solution and frozen at -80°C.

Immunoblot Analysis

Samples were separated on a gradient 8-16% SDS-PAGE, transferred to PVDF membranes and stained with either a monoclonal α-synuclein antibody (Transduction Labs, 1:1000) or a phospho-dependent tau monoclonal antibody (PHF-1 or AT8, 1:200) followed by anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and detected with SuperSignal™ West Pico Enhanced Chemiluminescence Kit (Pierce), followed by exposure to Kodak film.

Quantification of Immunoblots

Densitometry analysis was performed using ImageJ software. Bands were analyzed and normalized to actin levels. Unpaired student t tests were used to determine statistical significance between expression levels in the animals (n=5 for both the transgenic symptomatic and non-transgenic animals).

RESULTS

Symptomatic A30P mice exhibit synuclein and ubiquitin aggregates in the brainstem

As reported previously, mice over-expressing the A30P α-synuclein transgene developed symptoms between 6 – 14 months (Kahle et al., 2001). The clinical phenotype presented with tremor followed by increasing bradykinesia, first manifest as hind limb impairment. As the clinical phenotype progressed, the mice developed postural instability and ultimately paralysis. For most experiments, we sacrificed mice when they developed an end stage phenotype, which consisted of paralysis, postural instability and absence of grooming behavior. The clinical phenotype evolved over a time course of 3-4 weeks.

Mice exhibiting an end stage phenotype were sacrificed and their brains analyzed by biochemistry and immunoblotting. For the these studies, whole brain tissue was homogenized and protein aggregates were partially purified based on solubility in the presence of the detergent sarkosyl, as described previously (Kahle et al., 2001; Giasson et al., 2002; Lee et al., 2002). The samples were separated into 3 fractions: aqueous soluble, sarkosyl soluble and sarkosyl insoluble. All fractions showed more monomeric α-synuclein in the transgenic mice compared

to the non-transgenic mice. The sarkosyl insoluble fraction from symptomatic transgenic mice showed extensive higher molecular weight α-synuclein reactivity that was not apparent in sarkosyl insoluble fractions from either age-matched non-transgenic or asymptomatic transgenic mice (fig. 1A & B). These higher molecular weight bands likely represent oligomeric and aggregated α-synuclein as reported previously (Kahle et al., 2001; Giasson et al., 2002; Lee et al., 2002).

In order to determine where aggregated α-synuclein was present in the brain, we performed immunohistochemistry on sagitally-cut brain sections. Aggregated α-synuclein accumulated mainly in large neurons in the rostral part of the reticular formation and as punctate inclusions in the neuropil throughout the reticular formation of transgenic symptomatic mice (fig. 1C, panel a). α-Synuclein staining in age-matched non-transgenic mice was weaker and showed fewer inclusions (fig. 1C, panel d). α-Synuclein staining in large neurons of symptomatic transgenic mice showed strong diffuse expression (fig. 1C, panel a). These neurons also contained α-synuclein inclusions (fig. 1C, panels a & c, arrows). These inclusions also stained positively with anti-ubiquitin antibody indicating that they contain ubiquitin (fig. 1C, panels b & c, arrows). No reactivity was seen with pre-immune serum (fig. 1C, panel e).

Symptomatic A30P mice exhibit increased pathological phosphorylation of tau

Brain sections from mice exhibiting end-stage motor impairment (defined as tremor, muscular rigidity and full paralysis) were analyzed by immunoblot for the presence of abnormally phosphorylated tau with the phospho-specific antibody PHF-1 (Ser 396/404). Immunoblots of whole brain homogenates demonstrated over a 4 fold increase in PHF-1 reactivity in symptomatic transgenic mice compared to non-transgenic controls (fig. 2A & B). In addition, some of the PHF-1 reactive tau migrated at a higher molecular weight, which is characteristic of abnormally phosphorylated tau in diseased brain (fig. 2A). The total amount of tau as detected by the Tau-5 antibody, however, did not differ among the groups (fig. 2A & B). Abnormally phosphorylated tau in disease brain often shifts to an insoluble pool. To determine whether such a shift was apparent in the symptomatic transgenic mice, we performed stepwise solubility fractionation to separate salt soluble, sarkosyl soluble, and sarkosyl insoluble fractions. All brain tissue fractions of symptomatic transgenic mice showed increased PHF-1 signal

compared to age-matched non-transgenic mice as analyzed by PHF-1 immunoblot. A representative immunoblot of the sarkosyl insoluble fraction in Figure 2C shows increased S396 and S404 phosphorylation detected with the PHF-1 antibody in a symptomatic transgenic mouse. A similar pattern of reactivity was observed when the insoluble fraction was probed with the Tau -5 antibody, an antibody that recognizes tau from Arg221 to Leu282 and is independent of phosphorylation state (fig. 2C). The increased total tau reactivity observed in the insoluble fraction from symptomatic transgenic mice compared to control mice reflects the tendency of abnormally phosphorylated tau to shift to the sarkosyl insoluble fraction, which increases the total amount of tau in this fraction. Immunoblotting with anti-actin antibody confirmed equal protein loading among the different samples (fig. 2C). Further washing of the insoluble fraction did not remove either the PHF-1 positive or Tau5 positive tau, which indicates that the presence of the tau in the insoluble fraction was not due to carry-over of sample from the soluble fraction (data not shown).

Finally, we wanted to determine the relationship between abnormal tau phosphorylation and clinical symptoms. Therefore we performed immunoblots with PHF-1 antibody to compare sarkosyl-insoluble fractions of brain tissue from symptomatic transgenic (12 months old), aged-matched asymptomatic transgenic (14 months old), and aged-matched non-transgenic mice (14 months old). The immunoblot analysis showed increased phosphorylation of tau on serine residues 396 and 404 in the symptomatic mice compared to the non-transgenic mice, and little or no change in the non-symptomatic transgenic mice (fig. 2D).

To determine whether tau phosphorylation is found in the same brain regions as the synuclein pathology, we performed double label immunofluorescent analysis. Double labeling immunofluorescent analysis with antibodies to synuclein and phosphorylated tau (PHF-1, Ser396 and Ser404) demonstrated increased labeling in symptomatic animals compared to age-matched non-transgenic mice (Fig. 2B). The symptomatic mice exhibited many synuclein positive punctate inclusions in the ventral portion of the reticular formation (Fig 2B, panel d). Many neurons in this region exhibited diffuse cytoplasmic PHF-1 reactivity as well as punctate PHF-1 labeling within neurons (Fig 2B, panel e). The neurons that were diffusely positive for α-synuclein were large neurons located in the dorsal reticular formation, while the neurons that were diffusely positive for PHF-1 were small neurons located in the ventral reticular formation. A merge of the pictures that were diffusely positive for PHF-1 but contained punctate α-

synuclein reactivity showed that 48 ± 11% of the PHF-1-positive punctate tau inclusions co-localized with punctate α-synuclein inclusions (Fig 2B, panel f white arrows). The age matched non-transgenic mouse displayed weaker staining of synuclein and phosphorylated tau (Fig 2B, panels a, b).

To further characterize the pathological phosphorylation of tau in these animals, we utilized AT8, another phospho-tau antibody that recognizes tau phosphorylated on amino acids Ser199, Ser202, and Thr205 (Biernat et al., 1992). The results paralleled those seen with the PHF-1 antibody. Immunoblot analysis showed more reactivity in the sarkosyl insoluble fraction from the symptomatic transgenic mice than in the non- transgenic control mice and asymptomatic transgenic control mice (fig. 3A & B). In addition, tau from the symptomatic transgenic mice migrated at a slightly higher molecular weight (fig. 3A, AT8 and Tau-5 panels). The sarkosyl soluble fractions also showed increased AT8 signal in the symptomatic transgenic mice compared to non-transgenic mice (fig. 3A). No difference in actin reactivity was observed among the lanes demonstrating that there was equal protein among the samples (Fig 3A).

Immunofluorescent analysis with AT8 antibody also showed increased tau phosphorylation on residues 199, 202, 205 in small neurons of the pontine reticular formation of symptomatic transgenic mice (Fig 3B, panel e). Staining with an antibody to α-synuclein showed large neurons exhibiting diffuse anti-α-synuclein reactivity, as well as areas of punctate α-synuclein staining present both in the large neurons and throughout the neuropil (fig 3B, panel d). The fraction of AT8 positive neurons that also showed aggregated α-synuclein staining (as small punctate loci, rather than diffuse staining of the entire neuron) was 47 ± 10%. Furthermore, a small number of large, synuclein-positive neurons (14 ± 7%) also harbored small AT8 positive inclusions (Fig 3B, panel f, arrow heads). The non-transgenic animal exhibited weaker staining with both synuclein and AT8 (Fig 3B, panels a, b & c) and co-localization was not evident.

Increased activity of cyclin dependent kinase 5 in symptomatic transgenic mice

Two kinases are most commonly associated with PHF-1 reactivity, cyclin dependent kinase 5 (CDK5) and glycogen synthase kinase 3β (Paudel, 1997; Wang et al., 1998; Godemann et al., 1999). To determine whether either of these kinases are activated by mutant α-synuclein in these mice, tissue sections from symptomatic and non-symptomatic A30P α-synuclein mice

and aged matched non-transgenic control mice were probed with antibodies to activated forms of CDK5 and glycogen synthase kinase 3β. The immunoreactivity detecting activated CDK5 was increased in symptomatic A30P α-synuclein mice (fig. 5). The CDK5 reactivity presented as punctate loci of immunoreactivity apparent throughout the cytoplasm of some cells or as diffuse cytoplasmic reactivity present in other cells (fig. 5 A). A fraction of the reactive cells co-localized with PHF-1 reactivity (fig. 5 A-C). Basal levels of activated CDK5 immunoreactivity were present in tissue from both non-symptomatic (fig. 5D) and non-transgenic mice (fig. 5E). However the number of active CDK5-positive neurons per high-powered field in the brain stem was significantly higher in the symptomatic A30P α-synuclein transgenic mice compared to non-transgenic mice (fig. 5G, overall p value=0.0022). The number of active CDK5-positive neurons per high-powered field in the brain stem of symptomatic A30P α-synuclein mice was also greater than that of the non-symptomatic A30P α-synuclein mice, but this only reached a level of statistical significance indicative of a trend (p=0.07, fig. 5G). Immunoblots with antibody to activated CDK5 did not show any increase in reactivity, possibly because the number of cells showing increased reactivity is too small to appear in the gross analysis of tissue reactivity that occurs with an immunoblot (data not shown)). No increase in activity of glycogen synthase kinase 3β was apparent by immunoblotting or immunohistochemistry (data not shown).

Increased reactivity for glial fibrillary acidic protein in symptomatic transgenic mice

To assess other signs of injury we performed immunohistochemistry with an antibody directed against glial fibrillary (GFAP) acidic protein to detect astrocytosis, which is a classic marker for neuronal injury. We observed a massive increase in GFAP reactivity present throughout the brain stem of symptomatic A30P α-synuclein mice (fig. 6A & B) compared to non-symptomatic A30P α-synuclein transgenic mice (fig. 6F) or non-transgenic mice (fig. 6E & G). The amount of GFAP reactivity was not significantly increased in other brain areas, such as cortex (fig. 6A & E). The presence of the GFAP reactivity throughout contrasted sharply with the focal loci of reactivity seen with the phospho-tau or α-synuclein immunoreactivity (fig. 1B, 2B and 3B).

DISCUSSION

Transgenic mice over-expressing α-synuclein develop an age dependent accumulation of α-synuclein in neurons of the brain stem (Kahle et al., 2001; Giasson et al., 2002; Lee et al., 2002). Our study confirms the observations of α-synuclein aggregation observed by Kahle and colleagues, as well as by other groups studying mice over-expressing A53T or wild type α-synuclein. (Kahle et al., 2000; Masliah et al., 2000; Kahle et al., 2001; Giasson et al., 2002; Lee et al., 2002). We also observed the surprising finding that transgenic α-synuclein mice with impaired motor function accumulate tau that is phosphorylated at Ser202/Thr205 (detected with the AT8 antibody) and Ser396/Ser404 (detected with the PHF-1 antibody). Phosphorylation of tau at Ser202/Thr205 and Ser396/Ser404 is commonly observed in neurodegenerative diseases that are associated with tau pathology including FTDP-17, AD, and PSP (Jicha et al., 1997; Paudel, 1997; Wang et al., 1998; Godemann et al., 1999). Abnormal tau phosphorylation was readily apparent by both immunohistochemistry and immunoblot. Biochemical analyses of the brain homogenates indicate that hyper-phosphorylated tau was present in both sarkosyl soluble and the sarkosyl insoluble fractions of whole brain homogenates. Since the α-synuclein and tau pathology occur predominantly in the brain stem, it is likely that the increase in tau phoshorylation observed by immunoblot would even stronger if lysates from dissected brain stem were specifically examined by immunoblot. The presence of tau in the sarkosyl insoluble fraction, suggests that some of the tau is either self-aggregated or associated with another aggregate, such as aggregated α-synuclein. The presence of tau that contains pathological phosphorylation but lacks conformational epitopes associated with neurofibrillary tangles suggests that the tau exists at a stage characteristic of pre-tangles or early tangle formation. Incomplete tangle formation has been observed in aged mice expressing the human amyloid precursor protein APP 717 or Swedish mutations (Games et al., 1995; Hsiao et al., 1996). Progression to full tangle formation might not occur in mice because mouse tau does not appear to have the same tendency as human tau to aggregate in vivo (Lewis et al., 2000; Lewis et al., 2001; Tatebayashi et al., 2002).

One surprising aspect of tau pathology in the A30P α-synuclein mice is that it appears to form predominantly in neurons that did not overtly accumulate α-synuclein. This observation is consistent with recent studies of pathological tau and α-synuclein aggregates in human and mouse tissues. Tau can be detected in some Lewy bodies in PD substantia nigra, but many Lewy

bodies do not contain pathological tau (Ishizawa et al., 2003). Pathological tau can also be detected in brain tissue from human subjects who had PD caused by the A53T α-synuclein mutation as well as in transgenic mice co-expressing P301L tau and A53T α-synuclein, however the tau and α-synuclein are largely not co-localized (Duda et al., 2002; Giasson et al., 2003). These results are therefore consistent with our observation that only a small proportion of pathological tau that co-localizes with α-synuclein accumulations. The presence of pathological tau in neurons that do not show α-synuclein accumulation could result from the presence of small amounts of aggregated α-synuclein in these neurons, which might either directly stimulate the stress kinases or might stimulate aggregation of tau. Alternatively, the pathological tau might derive from extracellular stimuli emanating from neurons that have been injured by the accumulation of large amounts of α-synuclein. Neurons containing large amounts of α-synuclein accumulation might generate free radicals and or might fail to send out trophic signals required by neighboring neurons.

Other proteins known to be involved in the neurodegenerative pathology appear to accumulate along with the α-synuclein. Lewy bodies are composed primarily of the proteins α-synuclein and ubiquitin, but other proteins are present in some Lewy bodies in smaller amounts, such as parkin, Synphilin-1, SEPT4, torsin and neurofilament (Goldman and Yen, 1986; Wakabayashi et al., 2000; Choi et al., 2001; Chung et al., 2001; Sharma et al., 2001; Schlossmacher et al., 2002; Ihara et al., 2003). Interestingly, we did not observe increases in parkin associated with the α-synuclein accumulation (data not shown). Ubiquitin is the protein most commonly associated with Lewy bodies, and we observed that the A30P α-synuclein mice show concomitant accumulation of ubiquitin. The ubiquitin that accumulates appears to occur in only a subset of the neurons that accumulate α-synuclein as shown by double staining sections from the brain stem of affected mice using antibodies to both α-synuclein and ubiquitin. The lower prevalence of ubiquitin accumulations suggests that ubiquitin accumulation occurs after that of α-synuclein, and is consistent with a prior study indicating that the accumulation of α-synuclein precedes the accumulation of ubiquitin in these mice (Kahle et al., 2001). Astrocytosis is another common indicator of neuronal injury, and immunocytochemical staining for GFAP showed a massive increase in GFAP reactivity throughout the brain stem of symptomatic A30P

transgenic mice. The pattern of GFAP reactivity is striking because the distribution is much broader than that observed for α-synuclein accumulation or phospho-tau immunoreactivity. Because the human A30P α-synuclein transgene is driven by the neuron specific Thy1 promoter, the astrocytosis is likely to occur in response to the neuronal injury, which suggests that the neuronal injury is more widespread than is apparent by immunocytochemical analysis determined with antibodies to α-synuclein. Astrocytosis has been noted in another transgenic α-synuclein mouse model, in MPTP toxicity and in PD with dementia, but is not a prevalent feature of rotenone induced toxicity or of classic cases of Parkinson's disease (Mirza et al., 2000; Tsuchiya et al., 2002; Gomez-Isla et al., 2003; Muramatsu et al., 2003). The presence of astrocytosis in the α-synuclein transgenic mice points out an important difference between this transgenic mouse model of PD and actual PD in humans.

Our studies suggest that the mechanism of increased phosphorylation of tau may involve activation of CDK5. The kinases glycogen synthase kinase 3β, CDK5 and c-jun kinase are all activated by oxidative stress (Shaw et al., 1998; Nemoto et al., 2000; Strocchi et al., 2003). Both glycogen synthase kinase 3β and CDK5 phosphorylate tau at S396 and S404 (Paudel, 1997; Wang et al., 1998; Godemann et al., 1999). In our study the increased tau phosphorylation correlated with increased CDK5 activity and not via GSK-3β activation. Activation of CDK5 has been shown to correlate with tau phosphorylation in a number of studies. A recent study by Noble and colleagues demonstrated that activation of CDK5 increases tau phosphorylation, accumulation and aggregation *in vivo*, which suggests that activation of CDK5 in the A30P α-synuclein mice could also be driving the abnormal phosphorylation of tau (Noble et al., 2003). Hyperphosphorylation of tau is also associated with increased CDK5 activity in CNS neurons in Alzheimer disease and in Neimann-Pick's disease (Vincent et al., 1997; Bu et al., 2002). We did not observe activation of glycogen synthase kinase 3β, but further experiments are needed to provide a definitive conclusion about whether glycogen synthase kinase 3β is activated in response to the α-synuclein pathology. Understanding how aggregation of α-synuclein activates CDK5 and stimulates abnormal phosphorylation of tau in the A30P α-synuclein mice could provide important insights into the mechanism of toxicity of α-synuclein in the brain.

Increasing evidence suggests common mechanisms of neurodegeneration among different diseases. In Alzheimer's disease, Parkinson's disease, frontotemporal dementias and polyglutamine disorders such as Huntington's disease, protein aggregation appears to drive neurodegeneration. In each case, *in vitro* studies show that the proteins that accumulate have a inherent tendency to aggregate (DiFiglia et al., 1997; Conway et al., 1998; Hong et al., 1998; Hutton et al., 1998; Lewis et al., 2000). Many of these proteins have also been shown to stimulate concomitant aggregation of proteins prone to aggregate. Human tau aggregates as Aβ accumulates *in vivo*, Aβ stimulates aggregation of α-synuclein, α-synuclein co-aggregates with proteins containing expanded polyglutamine regions, and α-synuclein stimulates tau aggregation (Charles et al., 2000; Furlong et al., 2000; Lewis et al., 2001; Masliah et al., 2001; Giasson et al., 2003). In addition, the formation of protein aggregates appears to stimulate similar pathological reactions, including formation of free radicals, activation of stress kinases and inhibition of proteasomal activity. Because formation of micro-aggregates (also known as protofibrils) and activation of stress responses often occur in parallel, the exact mechanism of tau pathology in the A30P α-synuclein mice remains to be determined. However, our data provide *in vivo* support for the hypothesis that accumulation and aggregation of α-synuclein can stimulate pathological changes in tau protein.

Figure Legends

Figure 1: Accumulation of aggregated α-synuclein. A & B. The amount of aggregated α-synuclein observed in the sarkosyl insoluble fraction from symptomatic A30P transgenic mice (samples from a 14 month mouse shown) is much greater than the amount present in non-transgenic mice (A) or old asymptomatic transgenic A30P α-synuclein mice (B). C. Immunocytochemical analysis of a symptomatic A30P transgenic mouse shows neurons in the dorsal pons exhibiting both diffuse and punctate accumulation of α-synuclein (a). This region also shows an increase in ubiquitin immunoreactivity (b) that partly co-localizes with the α-synuclein reactivity (c, arrows). Less α-synuclein immunoreactivity is seen in the same region from an age matched non-transgenic α-synuclein mouse (d), and no α-synuclein immunoreactivity is seen in the same region from a symptomatic A30P transgenic mouse following pre-adsorption of antibody (e). Bar = 20 μm.

Figure 2: Induction of phosphorylated PHF-1-reactive tau protein. A. PHF-1 immunoblot of non-fractionated whole brain homogenates from transgenic symptomatic (+) or aged-matched non-transgenic mice. The blot was stripped and re-probed with tau-5 antibody which recognizes total tau, indicating similar total tau levels between animals. Actin immunoblot demonstrates equal protein loading. B. Quantification of differences in PHF-1 and total tau immunoreactivity in the homogenates. ImageJ densitometric analysis of the PHF-1 immunoreactivity normalized to actin levels in each animal (*p=.0035 in PHF-1, n=5) No significant difference in tau levels detected by Tau-5 existed between transgenic symptomatic and non-transgenic animals. C. Immunoblot of the sarkosyl insoluble fraction from a symptomatic A30P transgenic mouse shows increases in PHF-1 reactive tau protein compared to the same fraction from an aged matched non-transgenic mouse (arrow). Also note the electrophoretic shift in the PHF-1 immunoreactive band in the symptomatic transgenic mouse corresponding to increased phosphorylation (triangle). The lower panel shows actin immunoreactivity as a loading control. D. Comparison of the sarkosyl insoluble fraction from an aged non-transgenic mouse, an aged asymptomatic A30P α-synuclein transgenic mouse, and an aged symptomatic A30P α-synuclein transgenic mouse. The immunoblot shows that PHF-1-reactive tau increases only in the aged symptomatic transgenic mouse. The lower panel shows actin immunoreactivity as a loading

control. E. Immunocytochemical analysis of α-synuclein and PHF-1 immunoreactivity in the ventral pons. Staining of ventral pons from an aged non-transgenic mouse shows little α-synuclein (a, c) or PHF-1 (b, c) reactivity. In contrast staining of a symptomatic A30P transgenic mouse shows an increase in punctate α-synuclein staining (d), and both diffuse and punctate staining for PHF-1 (e). Merging of the pictures shows that some of the α-synuclein staining occurs in neurons containing the PHF-1 reactivity (f, arrows). Inset highlights α-synuclein and PHF-1 co-localization of a single neuron (yellow arrow). Bar = 20 μm.

Figure 3: Induction of phosphorylated AT8-reactive tau protein. A Immunoblot of fractionated samples in old (12 months) symptomatic A30P transgenic mouse and young (2 months) asymptomatic A30P transgenic mouse. AT8 antibody demonstrates increased phosphorylation of tau in the symptomatic mouse compared to the asymptomatic mouse. Tau-5 immunoreactivity of the same immunoblot shows a shift in tau mobility in the symptomatic mouse likely due to an increased phosphorylated tau protein which migrates more slowly on SDS PAGE B. Immunoblot of the sarkosyl insoluble fraction from a symptomatic A30P transgenic mouse shows the increase in AT8 reactive tau protein compared to the same fraction from aged matched non-transgenic mouse. Actin immunoblot confirms equal protein loading. C. Immunocytochemical analysis of an aged non-transgenic mouse shows little staining of α-synuclein (a, c) or AT8 (b, c). In contrast staining of a symptomatic A30P transgenic mouse shows an increase in punctate α-synuclein labeling (d), and neurons in the ventral pons with both diffuse and punctate accumulation of AT8 (e). Merging of the pictures shows that some of the α-synuclein staining occurs in neurons containing the AT8 reactivity (f, arrows). Bar = 20 μm.

Figure 4: CDK5 activity increases in symptomatic A30P α-synuclein mice. A-C. Immunoreactivity against activated CDK5 was apparent in the brain stem from a 6 month old symptomatic A30P α-synuclein mouse using an antibody against activated CDK5 (A, arrows). Double labeling with PHF-1 antibody (B) and showed co-localization of the two signals (C, arrows).The reactivity presented with a punctate or diffuse cytoplasmic reactivity. D & E. Little or no activated CDK5 reactivity was apparent in tissue sections from a 2-month old non-symptomatic A30P α-synuclein mouse (D) or a 12-month old non-transgenic mouse (E). No

reactivity was apparent with primary antibody omitted (F). (G) Quantification of the reactivity with the antibody against activated CDK5 showed that the number of positive neurons was significantly higher in symptomatic A30P transgenic mice compared to non-symptomatic A30P transgenic mice and non-transgenic mice ($N=3$, $p<0.005$). 10 High-powered fields were counted per mouse in 3 mice from each group. A two way ANOVA was used with an F value of 21.48 and $p < 0.0001$. Bar = 20 μm.

Figure 5: GFAP activity increases in symptomatic A30P α-synuclein mice. A & B. Abundant immunoreactivity against GFAP was apparent in the brain stem from a 12-month-old symptomatic A30P α-synuclein mouse using an antibody against activated GFAP (A, 2x; B, 40x). C & D. Double labeling with PHF-1 antibody showed separation of localization of the PHF-1 (C) and GFAP reactivity (D). Little GFAP reactivity was apparent in tissue sections from a 4-month old non-symptomatic A30P α-synuclein mouse (F, 40x), a 12-month old non-transgenic mouse (E, 2x; G, 40x) or symptomatic A30P α-synuclein mouse with anti-GFAP antibody omitted (H). Bar: B-D & F-H=20 μm.

REFERENCES

Biernat J, Mandelkow EM, Schroter C, Lichtenberg-Kraag B, Steiner B, Berling B, Meyer H, Mercken M, Vandermeeren A, Goedert M, et al. (1992) The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. Embo J 11:1593-1597.

Bu B, Li J, Davies P, Vincent I (2002) Deregulation of cdk5, hyperphosphorylation, and cytoskeletal pathology in the Niemann-Pick type C murine model. J Neurosci 22:6515-6525.

Charles V, Mezey E, Reddy PH, Dehejia A, Young TA, Polymeropoulos MH, Brownstein MJ, Tagle DA (2000) Alpha-synuclein immunoreactivity of huntingtin polyglutamine aggregates in striatum and cortex of Huntington's disease patients and transgenic mouse models. Neurosci Lett 289:29-32.

Choi P, Golts N, Snyder H, Petrucelli L, Chong M, Hardy J, Sparkman D, Cochran E, Lee J, Wolozin B (2001) Co-association of parkin and α-synuclein. NeuroReport 12:2839-2844.

Chung K, Zhang Y, Lim K, Tanaka Y, Huang H, Gao J, Ross C, Dawson V, Dawson T (2001) Parkin Ubiquitinates the α-Synuclein-Interacting Protein, Synphilin-1: Implications for Lewy Body Formation in Parkinson's Disease. Nature Med 7:1144-1150.

Conway K, Harper J, Lansbury P (1998) Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nature Med 4:1318-1320.

DiFiglia M, Sapp E, Chase K, Davies S, Bates G, Vonsattel J, Aronin N (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277:1990-1993.

Duda JE, Giasson BI, Mabon ME, Miller DC, Golbe LI, Lee VM, Trojanowski JQ (2002) Concurrence of alpha-synuclein and tau brain pathology in the Contursi kindred. Acta Neuropathol (Berl) 104:7-11.

Engelender S, Kaminsky Z, Guo X, Sharp A, Amaravi R, Kleiderlein J, Margolis R, Troncoso J, Lanahan A, Worley P, Dawson V, Dawson T, Ross C (1999) Synphilin-1 associates with alpha-synuclein and promotes the formation of cytosolic inclusions. Nature Gen 22:110-114.

Feany MB, Bender WW (2000) A Drosophila model of Parkinson's disease. Nature 404:394-398.

Forman MS, Schmidt ML, Kasturi S, Perl DP, Lee VM, Trojanowski JQ (2002) Tau and alpha-synuclein pathology in amygdala of Parkinsonism-dementia complex patients of Guam. Am J Pathol 160:1725-1731.

Furlong RA, Narain Y, Rankin J, Wyttenbach A, Rubinsztein DC (2000) alpha-synuclein overexpression promotes aggregation of mutant huntingtin. Biochem J 346 Pt 3:577-581.

Games D, Adams D, Alessandrini R, Barbour R, Berthelette, Blackwell C, Carr T, Clemens J, Donaldson T, Gillespie F, Guido T, Hagopian S, Johnson M, Wood K, Khan K, Lee M, Leibowitz P, Lieberburg I, Little S, Masliah E, McConlogue L, Montoya-Zavala M, Mucke L, Paganini L, Penniman E, Power M, Schenk D, Seubert P, Snyder B, Soriano F, Tan H, Vitale J, Wadsworth S, Wolozin B, Zhao J (1995) Development of neuropathology similar to Alzheimer's disease in transgenic mice overexpressing the 717_{V-F} ß-amyloid precursor protein. Nature 373:523-527.

Giasson B, Duda J, Quinn S, Zhang B, Trojanowski J, Lee V-Y (2002) Neuronal α-synucleinopathy with severe movement disorder in mice expressing A53T human α-synuclein. Neuron 34:521-533.

Giasson BI, Forman MS, Higuchi M, Golbe LI, Graves CL, Kotzbauer PT, Trojanowski JQ, Lee VM (2003) Initiation and synergistic fibrillization of tau and alpha-synuclein. Science 300:636-640.

Godemann R, Biernat J, Mandelkow E, Mandelkow EM (1999) Phosphorylation of tau protein by recombinant GSK-3beta: pronounced phosphorylation at select Ser/Thr-Pro motifs but no phosphorylation at Ser262 in the repeat domain. FEBS Lett 454:157-164.

Goldman JE, Yen SH (1986) Cytoskeletal Protein Abnormalities in Neurodegenerative Diseases. Ann Neurol 19:209-223.

Gomez-Isla T, Irizarry MC, Mariash A, Cheung B, Soto O, Schrump S, Sondel J, Kotilinek L, Day J, Schwarzschild MA, Cha JH, Newell K, Miller DW, Ueda K, Young AB, Hyman BT, Ashe KH (2003) Motor dysfunction and gliosis with preserved dopaminergic markers in human alpha-synuclein A30P transgenic mice. Neurobiol Aging 24:245-258.

Hashimoto M, LJ H, Xia Y, Takeda A, Sisk A, Sundsmo M, Masliah E (1999) Oxidative stress induces amyloid-like aggregate formation of NACP/α-synuclein *in vitro*. NeuroReport 10:717-721.

Hong M, Zhukareva V, Vogelsberg-Ragaglia V, Wszolek Z, Reed L, Miller B, Geschwind D, Bird T, McKeel D, Goate A, Morris J, Wilhelmsen K, Schellenberg G, Trojanowski J, Lee V (1998) Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. Science 282:1914-1917.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice. Science 274:99-102.

Hutton M, Lendon C, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, Hackett J, Adamson J, Lincoln S, Dickson D, Davies P, Petersen R, Stevens M, de Graaff E, Wauters E, van Baren J, Hillebrand M, Joosse M, Kwon J, Nowotny P, Heutink P, al e (1998) Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393:702-705.

Ihara M, Tomimoto H, Kitayama H, Morioka Y, Akiguchi I, Shibasaki H, Noda M, Kinoshita M (2003) Association of the cytoskeletal GTP-binding protein Sept4/H5 with cytoplasmic inclusions found in Parkinson's disease and other synucleinopathies. J Biol Chem.

Ishizawa T, Mattila P, Davies P, Wang D, Dickson DW (2003) Colocalization of tau and alpha-synuclein epitopes in Lewy bodies. J Neuropathol Exp Neurol 62:389-397.

Jenco J, Rawlingson A, Daniels B, Morris A (1998) Regulation of phospholipase D2: Selective inhibition of mammalian phospholipase D isoenzymes by α and β synucleins. Biochemistry 37:4901-4909.

Jensen PH, Hager H, Nielsen MS, Hojrup P, Gliemann J, Jakes R (1999) alpha-synuclein binds to Tau and stimulates the protein kinase A- catalyzed tau phosphorylation of serine residues 262 and 356. J Biol Chem 274:25481-25489.

Jicha GA, Lane E, Vincent I, Otvos L, Jr., Hoffmann R, Davies P (1997) A conformation- and phosphorylation-dependent antibody recognizing the paired helical filaments of Alzheimer's disease. J Neurochem 69:2087-2095.

Kahle PJ, Neumann M, Ozmen L, Muller V, Jacobsen H, Schindzielorz A, Okochi M, Leimer U, van Der Putten H, Probst A, Kremmer E, Kretzschmar HA, Haass C (2000) Subcellular

localization of wild-type and Parkinson's disease- associated mutant alpha -synuclein in human and transgenic mouse brain. J Neurosci 20:6365-6373.

Kahle PJ, Neumann M, Ozmen L, Muller V, Odoy S, Okamoto N, Jacobsen H, Iwatsubo T, Trojanowski JQ, Takahashi H, Wakabayashi K, Bogdanovic N, Riederer P, Kretzschmar HA, Haass C (2001) Selective insolubility of alpha-synuclein in human Lewy body diseases is recapitulated in a transgenic mouse model. Am J Pathol 159:2215-2225.

Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen J, Schols L, Riess O (1998) Ala30Pro mutation in the gene encoding α-synuclein in Parkinson's disease. Nature Gen 18:106-108.

Lee FJ, Liu F, Pristupa ZB, Niznik HB (2001) Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. Faseb J 15:916-926.

Lee M, Stirling W, Xu Y, Xu X, Qui D, Mandir A, Dawson T, Copeland N, Jenkins N, Price D (2002) Human α-synuclein-harboring familial Parkinson's disease-linked Ala-53 to Thr mutation causes neurodegenerative disease with α-synuclein aggregation in transgenic mice. PNAS 99.

Lewis J, Dickson DW, Lin WL, Chisholm L, Corral A, Jones G, Yen SH, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E (2001) Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP. Science 293:1487-1491.

Lewis J, McGowan E, Rockwood J, Melrose H, Nacharaju P, Van Slegtenhorst M, Gwinn-Hardy K, Paul Murphy M, Baker M, Yu X, Duff K, Hardy J, Corral A, Lin WL, Yen SH, Dickson DW, Davies P, Hutton M (2000) Neurofibrillary tangles, amyotrophy and progressive motor disturbance in mice expressing mutant (P301L) tau protein. Nat Genet 25:402-405.

Masliah E, Rockenstein E, Veinbergs I, Sagara Y, Mallory M, Hashimoto M, Mucke L (2001) beta -Amyloid peptides enhance alpha -synuclein accumulation and neuronal deficits in a transgenic mouse model linking Alzheimer's disease and Parkinson's disease. Proc Natl Acad Sci U S A 25:25.

Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A, Mucke L (2000) Dopaminergic Loss and Inclusion Body Formation in alpha-Synuclein Mice: Implications for Neurodegenerative Disorders. Science 287:1265-1269.

Mirza B, Hadberg H, Thomsen P, Moos T (2000) The absence of reactive astrocytosis is indicative of a unique inflammatory process in Parkinson's disease. Neuroscience 95:425-432.

Muramatsu Y, Kurosaki R, Watanabe H, Michimata M, Matsubara M, Imai Y, Araki T (2003) Expression of S-100 protein is related to neuronal damage in MPTP-treated mice. Glia 42:307-313.

Murphy DD, Rueter SM, Trojanowski JQ, Lee VM (2000) Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. J Neurosci 20:3214-3220.

Nemoto S, Takeda K, Yu ZX, Ferrans VJ, Finkel T (2000) Role for mitochondrial oxidants as regulators of cellular metabolism. Mol Cell Biol 20:7311-7318.

Noble W, Olm V, Takata K, Casey E, Mary O, Meyerson J, Gaynor K, LaFrancois J, Wang L, Kondo T, Davies P, Burns M, Veeranna, Nixon R, Dickson D, Matsuoka Y, Ahlijanian M, Lau LF, Duff K (2003) Cdk5 is a key factor in tau aggregation and tangle formation in vivo. Neuron 38:555-565.

Ostrerova N, Petrucelli L, Farrer M, Mehta N, Alexander P, Choi P, Palacino J, Hardy J, Wolozin B (1999) α-Synuclein shares physical and functional homology with 14-3-3 proteins. J Neurosci 19:5782-5791.

Ostrerova-Golts N, Petrucelli L, Hardy J, Lee J, Farrer M, Wolozin B (2000) The A53T α-Synuclein Mutation Increases Iron-Dependent Aggregation and Toxicity. J Neuroscience 20:6048-6054.

Paik S, Shin H, Lee J, Chang C, Kim J (1999) Copper(II)-induced self-oligomerization of α-synuclein. Biochem J 340:821-828.

Paik SR, Shin HJ, Lee JH (2000) Metal-catalyzed oxidation of alpha-synuclein in the presence of Copper(II) and hydrogen peroxide. Arch Biochem Biophys 378:269-277.

Paudel HK (1997) Phosphorylation by neuronal cdc2-like protein kinase promotes dimerization of Tau protein in vitro. J Biol Chem 272:28328-28334.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045-2047.

Pronin AN, Morris AJ, Surguchov A, Benovic JL (2000) Synucleins are a novel class of substrates for G protein-coupled receptor kinases. J Biol Chem.

Sahara N, Lewis J, DeTure M, McGowan E, Dickson DW, Hutton M, Yen SH (2002) Assembly of tau in transgenic animals expressing P301L tau: alteration of phosphorylation and solubility. J Neurochem 83:1498-1508.

Schlossmacher MG, Frosch MP, Gai WP, Medina M, Sharma N, Forno L, Ochiishi T, Shimura H, Sharon R, Hattori N, Langston JW, Mizuno Y, Hyman BT, Selkoe DJ, Kosik KS (2002) Parkin localizes to the lewy bodies of Parkinson disease and dementia with lewy bodies. Am J Pathol 160:1655-1667.

Sharma N, Hewett J, Ozelius LJ, Ramesh V, McLean PJ, Breakefield XO, Hyman BT (2001) A close association of torsinA and alpha-synuclein in Lewy bodies: a fluorescence resonance energy transfer study. Am J Pathol 159:339-344.

Sharon R, Goldberg MS, Bar-Josef I, Betensky RA, Shen J, Selkoe DJ (2001) alpha -Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins. Proc Natl Acad Sci U S A 98:9110-9115.

Shaw M, Cohen P, Alessi DR (1998) The activation of protein kinase B by H2O2 or heat shock is mediated by phosphoinositide 3-kinase and not by mitogen-activated protein kinase-activated protein kinase-2. Biochem J 336 (Pt 1):241-246.

Souza JM, Giasson BI, Lee VM, Ischiropoulos H (2000) Chaperone-like activity of synucleins. FEBS Lett 474:116-119.

Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. Proc Natl Acad Sci USA 95:6469-6473.

Strocchi P, Pession A, Dozza B (2003) Up-regulation of cDK5/p35 by oxidative stress in human neuroblastoma IMR-32 cells. J Cell Biochem 88:758-765.

Tatebayashi Y, Miyasaka T, Chui DH, Akagi T, Mishima K, Iwasaki K, Fujiwara M, Tanemura K, Murayama M, Ishiguro K, Planel E, Sato S, Hashikawa T, Takashima A (2002) Tau filament formation and associative memory deficit in aged mice expressing mutant (R406W) human tau. Proc Natl Acad Sci U S A 99:13896-13901.

Tsuchiya K, Ikeda K, Niizato K, Watabiki S, Anno M, Taki K, Haga C, Iritani S, Matsushita M (2002) Parkinson's disease mimicking senile dementia of the Alzheimer type: a clinicopathological study of four autopsy cases. Neuropathology 22:77-84.

Vincent I, Jicha G, Rosado M, Dickson DW (1997) Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. J Neurosci 17:3588-3598.

Wakabayashi K, Engelender S, Yoshimoto M, Tsuji S, Ross CA, Takahashi H (2000) Synphilin-1 is present in Lewy bodies in Parkinson's disease. Ann Neurol 47:521-523.

Wang JZ, Wu Q, Smith A, Grundke-Iqbal I, Iqbal K (1998) Tau is phosphorylated by GSK-3 at several sites found in Alzheimer disease and its biological activity markedly inhibited only after it is prephosphorylated by A-kinase. FEBS Lett 436:28-34.

Figure 1

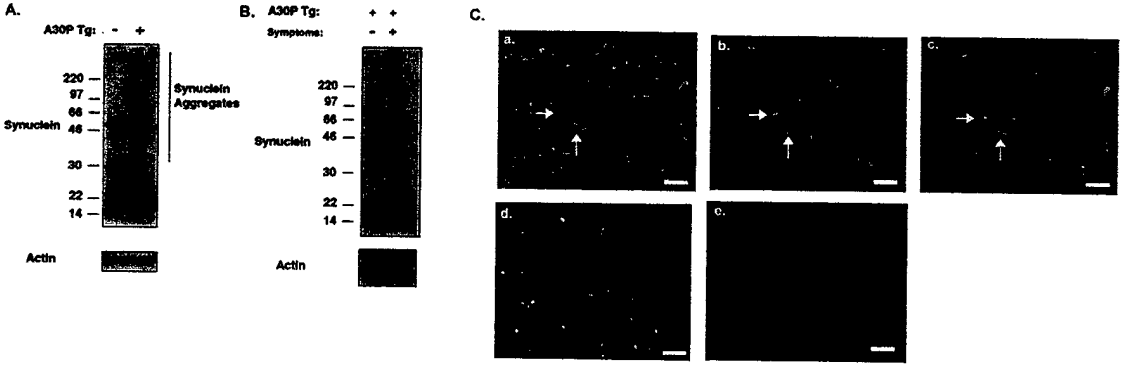

A.
A30P Tg: - +
220
97
66
46
30
22
14
Synuclein
Synuclein Aggregates
Actin
B.
A30P Tg: + +
Symptoms: - +
220
97
66
46
30
22
14
Synuclein
Actin
C.
a.
b.
c.
d.
e.

Figure 2

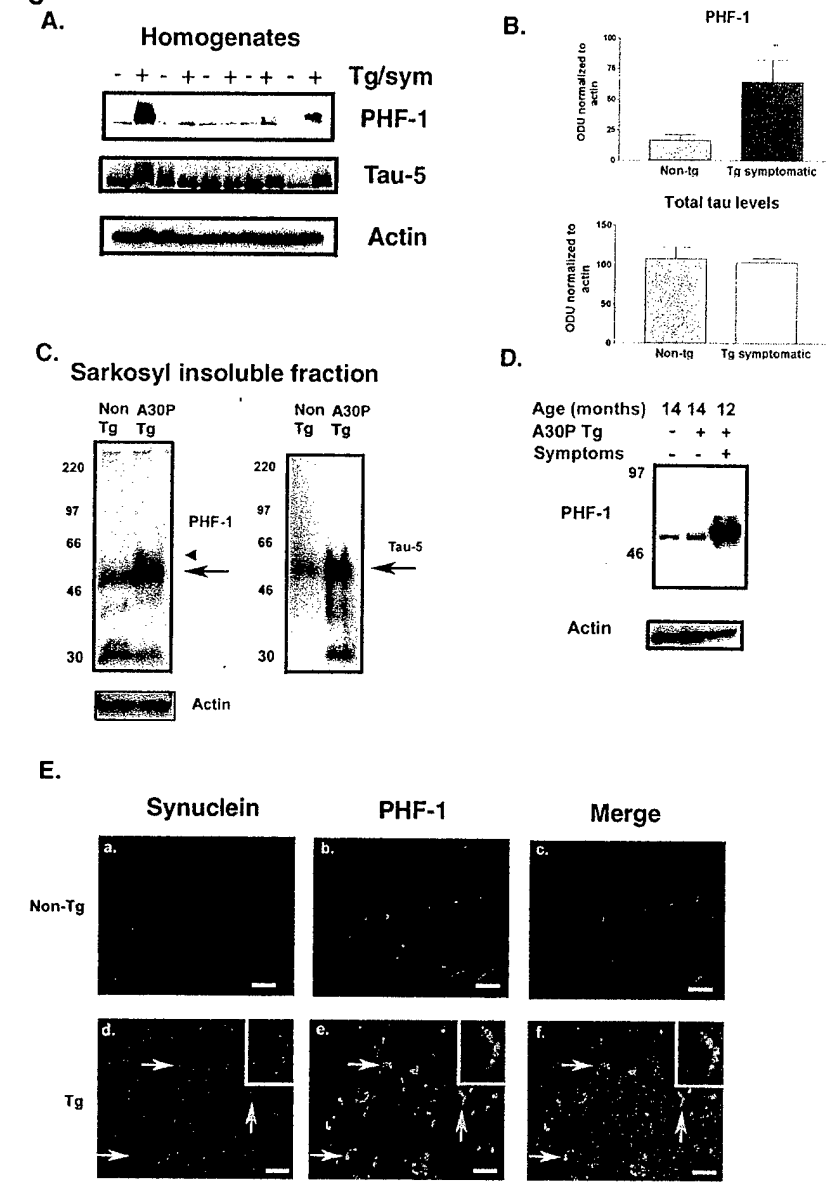
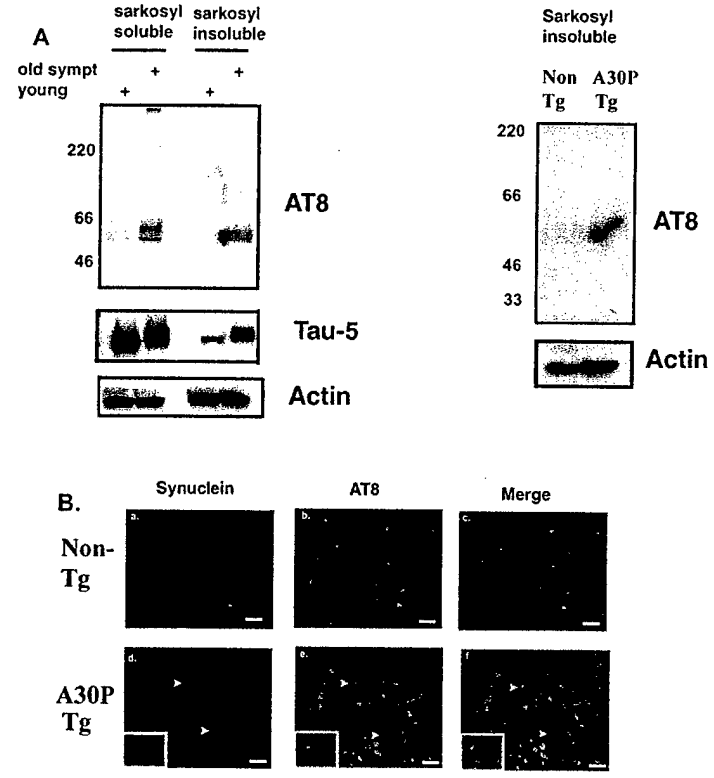

A.
Homogenates
- + - + - + - + Tg/sym
PHF-1
Tau-5
Actin
B.
PHF-1
ODU normalized to actin
Non-tg
Tg symptomatic
Total tau levels
ODU normalized to actin
Non-tg
Tg symptomatic
C.
Sarkosyl insoluble fraction
Non Tg
A30P Tg
220
97
66
46
30
PHF-1
Tau-5
Actin
D.
Age (months) 14 14 12
A30P Tg - + +
Symptoms - - +
97
46
PHF-1
Actin
E.
Synuclein
PHF-1
Merge
Non-Tg
Tg
a.
b.
c.
d.
e.
f.

Figure 3


A
sarkosyl soluble
sarkosyl insoluble
old sympt
young
220
66
46
AT8
Tau-5
Actin
Sarkosyl insoluble
Non Tg
A30P Tg
220
66
46
33
AT8
Actin
B.
Synuclein
AT8
Merge
Non-Tg
A30P Tg

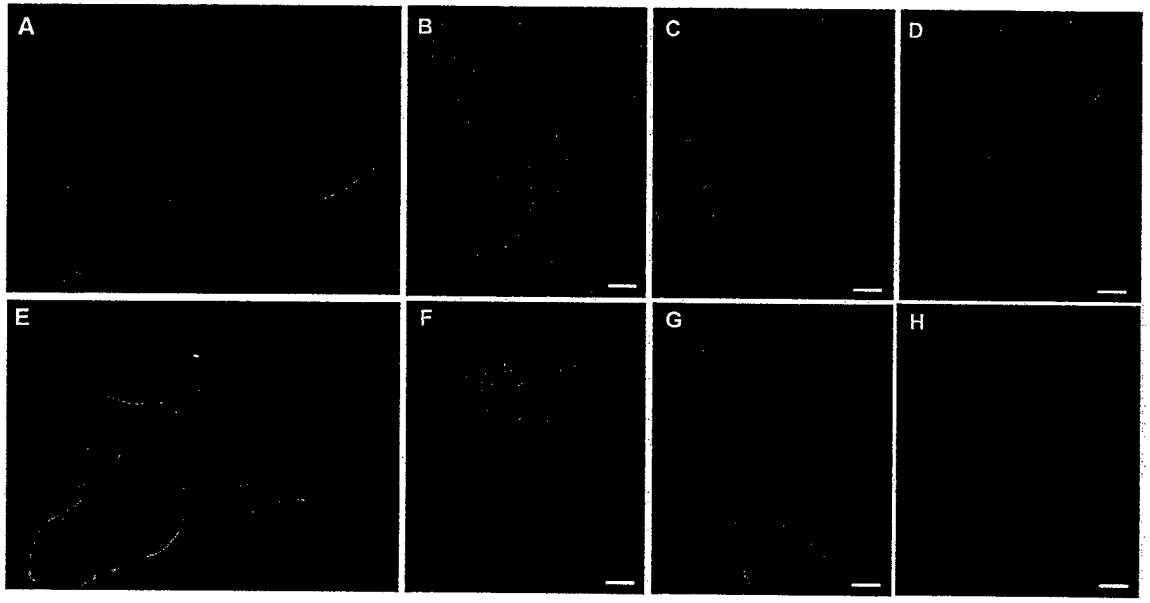
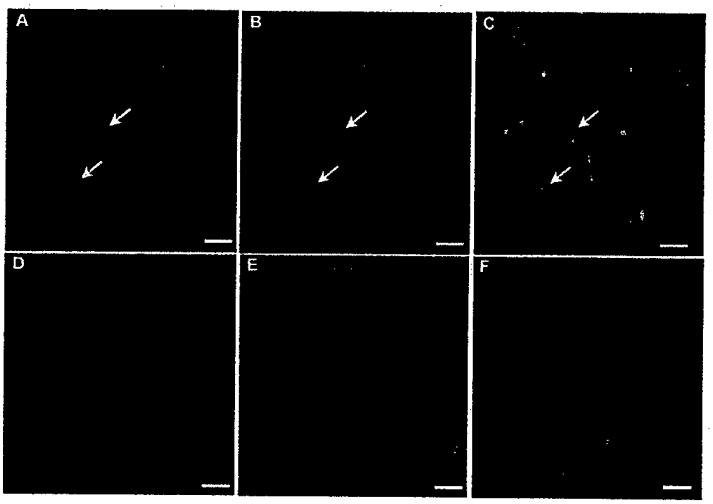
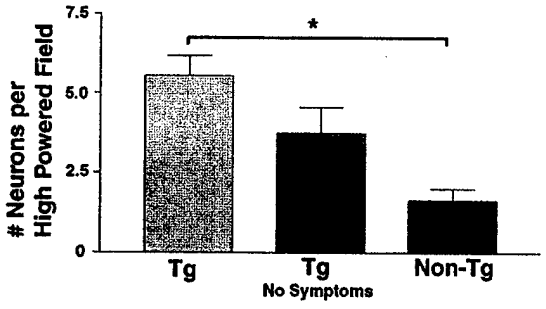

A
B
C
D
E
F
G
H

Figure 5


A
B
C
D
E
F

G.


Neurons per High Powered Field
7.5
5.0
2.5
0
*
Tg
Tg
No Symptoms
Non-Tg